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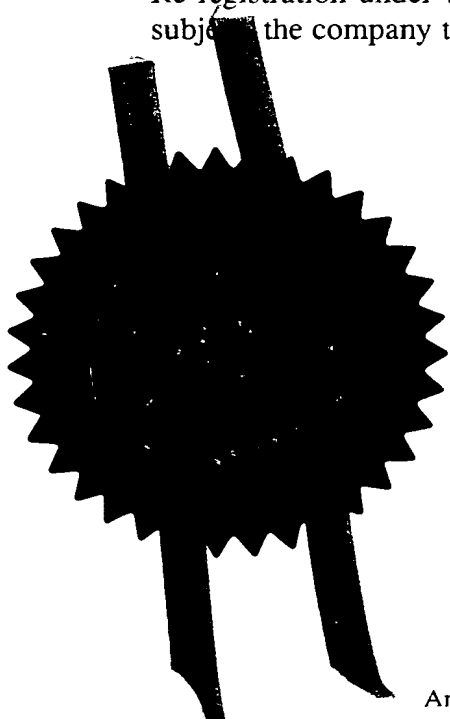
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2. Patent application number 25 JUN 1999
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THE UNIVERSITY OF BUCKINGHAM
HUNTER STREET
BUCKINGHAM
MK18 1EG
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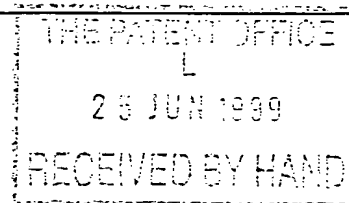
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4. Title of the invention METHODS AND COMPOSITIONS RELATING TO PANCREATIC ISLET AND β -CELL DYSFUNCTION

5. Name of your agent (if you have one) MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

YORK HOUSE
23 KINGSWAY
LONDON
WC2B 6HP



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CONTINUATION SHEET

3. Full name, address and postcode of the or of
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UNIVERSITY OF GENEVA
1 RUE MICHEL SERVET
CH1211
GENEVA 4
SWITZERLAND

7686328 cc1

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Methods and Compositions Relating to Pancreatic Islet and
 β -Cell Dysfunction

Field of the Invention

5 The present invention relates to methods and compositions relating to pancreatic islet and β -cell dysfunction, in conditions such as non-insulin dependent diabetes. Specifically, the present invention identifies and describes proteins that are differentially expressed in
10 non-insulin dependent diabetes relative to their expression in the normal state and, in particular, identifies and describes proteins associated with pancreatic islet or β -cell dysfunction and/or mass. Further, the present invention identifies and describes
15 proteins via their ability to interact with gene products involved in the regulation of pancreatic islets or β -cell dysfunction and/or mass. Still further, the present invention provides methods, particularly experimental paradigms, for the identification of differential
20 expressed proteins that are potential molecular targets for compounds to treat or prevent pancreatic islets or β -cell dysfunction and non-insulin dependent diabetes mellitus. Still further, the present invention provides methods for the identification and therapeutic use of
25 compounds for the prevention and treatment of pancreatic islet or β -cell dysfunction and non-insulin dependent diabetes mellitus.

Background of the Invention

30 Diabetes mellitus is one of the most common metabolic disorders affecting more than 100 million people worldwide (King, H. and Zimmet, P. (1988) World Health Statistics Quarterly 41, 190-196; Harris, M.I. et al (1992) Diabetes Care 15, 815-819). It is predicted that

the world incidence of diabetes will double by year 2010 largely through an increase in incidence in industrially developing countries such as India, China, South America and the Far East.

5

There are two types of diabetes. Type I, or insulin dependent diabetes mellitus (IDDM), is the result of progressive autoimmune destruction of the pancreatic β -cells and constitutes 5-10% of the total diabetic population. Type II diabetes, or non-insulin dependent diabetes mellitus (NIDDM) or maturity onset diabetes, represents 90-95% of the diabetic population. Typically it occurs in middle-aged and elderly subjects, although it can develop in younger subjects and it is commonly associated with obesity. NIDDM is associated with two metabolic defects: insulin resistance and inappropriate insulin secretion. The insulin resistance affects muscle and adipose tissues, resulting in reduced glucose uptake and elevated hepatic production of glucose. The pancreatic lesion is an essential component of NIDDM. Typically the initial lesion appears to be an over-production of insulin in response to a glucose load, but later there is a loss or reduction in the first phase insulin secretion response and eventually the insulin secretion capacity declines to such an extent that exogenous insulin needs to be administered. Family studies indicate a major genetic component in the development of NIDDM, but apart from a sub-type of NIDDM called maturity onset diabetes of the young (MODY), few susceptibility genes have been identified.

Traditionally treatment for non-insulin dependent diabetes mellitus has focussed on the control of blood

glucose. Current drugs, however, generally fail to achieve the same degree of control of blood glucose as is present in a non-diabetic subject. In addition, NIDDM patients often have elevated plasma triglycerides and elevated plasma cholesterol or a low ratio of HDL:LDL-cholesterol. All of these metabolic changes are adverse with respect to the development of the secondary complications of diabetes, which includes cardiovascular disease, blindness, nephropathy, stroke and microvascular disease.

There remains a pressing need in the art for more effective treatments for non-insulin dependent diabetes.

The diabetic population is extremely heterogenous and it is likely that the development of β -cell dysfunction and NIDDM has multiple causes. In part, it probably relates to a failure to meet the increased insulin requirement and arises as a result of insulin resistance. However, other factors including toxins, viral disease and genetically inherited defects cannot be ruled out.

There are a number of animal models with mutations that are associated with insulin resistance and attempts have been made to utilise such animals as models for the study of non-insulin dependent diabetes, insulin resistance and pancreatic β -cell function. The best studied animal models for insulin resistance and non-insulin dependent diabetes are mice, which contain the autosomal recessive mutations ob/ob (obese) and db/db (diabetes). These mutations are on chromosome 6 and 4 respectively, but lead to clinically similar pictures provided the genes are expressed on the same background strain. The ob gene

product has been identified as 16kDa polypeptide produced primarily by adipose tissue that provides a signal to the brain on the adipose tissue fat stores. Mice with a mutation, resulting in no circulating protein (called
5 leptin) are hyperphagic, obese, have poor thermoregulation and non-shivering thermogenesis and are insulin resistant with impaired glucose tolerance. When the OB gene mutation is on the C57Bl/6 background the mice do not present with frank clinically evident diabetes,
10 but are massively hyperinsulinaemic and glucose intolerant.

The db/db mice have a mutation in the receptor for leptin so that normal signal transduction via the JAK/STAT
15 pathway does not occur. This mutation, when on the C57Bl/6 background, is phenotypically identical to the ob mutation. However, the db/db mutation is normally expressed on the C57Bl/Ks mouse background and on this background the mutation causes frank diabetes. It is
20 recognised that a major difference between wild-type mice of the C57Bl/6 strain and wild-type mice of the C57Bl/Ks strain is that the Bl/6 mice have approximately twice the pancreatic islet cell mass of the Bl/Ks mice. Thus, mice of the C57Bl/6 strain are able to withstand the
25 pancreatic β -cell stress imposed by insulin resistance more than mice of the C57Bl/Ks strain.

Other mutant animal models include fa/fa (fatty) rats and ZDF fatty rats, which bear strong respective similarities
30 with the ob/ob and db/db mice. Thus the fa/fa rat is obese, insulin resistant, very hyperinsulinaemic and glucose intolerant, whereas the ZDF rat is obese, insulin resistant and hyperinsulinaemic, but the male rats

develop frank diabetes after approximately 6 weeks of age. It follows that the pancreatic islets of the Zucker fa/fa rats and ZDF male rats must differ in their ability to maintain insulin secretion in the face of insulin resistance. Similarly, pancreatic islets from ZDF male rats must be intrinsically different from pancreatic islets from ZDF female rats, which do not develop frank diabetes.

Inbred mouse strains, such as the NZO mouse, the Japanese KK mouse and the GK rat are models of insulin resistance, pancreatic β -cell dysfunction and diabetes. Further, desert rodents, such as spiny mice and sand rats are neither insulin resistant nor diabetic in their natural habitats, but do present frank diabetes when fed on a standard laboratory diet.

Insulin resistance, developing pancreatic β -cell dysfunction and glucose intolerance are a common feature of elderly rodents and the development of the defects can be accelerated by feeding diets with a high fat content, whether these diets are synthetic homogenous diets or are the result of supplementation or replacement of the normal rat chow by human food with a high fat content (cafeteria diet).

Human non-insulin dependent diabetes has a high concordance between twins with a higher rate in identical twins than in non-identical twins. There is also a strong familial tendency associated with both parents. Thus, offspring where both parents have NIDDM have a higher risk of developing NIDDM than offspring in which only one parent has NIDDM.

Recent data has shown that there is also a significant correlation between low birth-weight (in relation to gestational age) and the development of non-insulin dependent diabetes in later life. This suggests a possible linkage between in utero nutrition and the development of non-insulin dependent diabetes. The molecular nature of this linkage in human NIDDM has not been defined. However, studies in rats have shown that if pregnant female rats are fed on a 6% protein diet rather than the more usual 15% protein diet, the two sets of pregnant rats produce a similar number of offspring but the pups from the mothers fed on a 6% protein diet are smaller. If these pups are subsequently given post-weaning a high-fat diet to induce insulin resistance, then they develop non-insulin dependent diabetes. Furthermore, it has been demonstrated that the pups from the mothers fed on a 6% protein diet have a smaller islet cell mass than pups from mothers fed on a 15% protein diet.

Much of the current increase in incidence in NIDDM is occurring in developing countries. In such countries it is likely that the incidence of small for gestational age babies will be high. As these countries change from their traditional high carbohydrate, locally grown diet to a more western style high fat diet, as occurs during acculturalisation, the development of insulin resistance in subjects puts increased pressure on pancreatic islet function. The identification of those factors that are associated with the development of a low islet cell mass would allow the discovery of novel agents to prevent the development of non-insulin dependent diabetes as well as treat the pre-existing condition.

Absolute pancreatic β -cell mass is largely determined at birth or shortly after birth. However, the pancreatic β -cell mass is increased in certain situations, for example during pregnancy. Thus a mode for examining the molecular nature of proliferative activity of the pancreatic β -cell is the pregnant animal.

The above animal models have been used from time to time to evaluate new drugs that were potential treatments for pancreatic β -cell dysfunction or non-insulin dependent diabetes. However, although individual changes in enzyme activities have been identified in some of the animal models and how this might be altered by a drug therapy, no systematic evaluation has been made of the differences in protein expression in the pancreatic islet or β -cells of normal animals showing β -cell dysfunction. It is these changes in protein expression that underlie the development of β -cell dysfunction leading to non-insulin dependent diabetes. It is the same changes in protein expression that are likely to be causative of non-insulin dependent diabetes in humans and in companion animals such as dogs and cats. Given the severity and prevalence of non-insulin dependent diabetes and pancreatic β -cell dysfunction, there exists a great need for the systematic identification of the disease causing proteins, since modulation of the expression level of such proteins back to the level in non-diabetic individuals represents a means of treating the disease condition.

Summary of the Invention

Broadly, the present invention relates to methods and compositions for the treatment of pancreatic islet and β -cell dysfunction, including but not limited to, non-

insulin dependent diabetes. More specifically, the present invention identifies and describes proteins that are differentially expressed in then pancreatic β -cell or islets of Langerhans of animals exhibiting pancreatic β -cell dysfunction relative to their expression in normal animals and also identifies proteins that are differentially expressed in response to manipulations relevant to altering pancreatic β -cell mass or function. Such differentially expressed proteins (DEPs) may represent 'target proteins' and/or fingerprint proteins. Further, the present invention identifies and describes proteins termed pathway proteins via their ability to interact with proteins involved in the regulation of pancreatic islet and/or β -cell function. Pathway proteins may also exhibit target protein and/or fingerprint protein characteristics.

Accordingly, in a first aspect, the present invention provides a method of screening an agent to determine its usefulness in treating a condition characterised by pancreatic islet or β -cell dysfunction, the method comprising:

(a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of pancreatic islet or β -cell function;

(b) obtaining a sample of relevant tissue taken from, or representative of, a subject having reduced pancreatic islet or β -cell function, who or which has been treated with the agent being screened;

(c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the tissue from, or representative of, the

treated subject; and,

(d) selecting or rejecting the agent according to the extent to which it changes the expression of the differentially expressed protein or proteins in the treated subject having reduced pancreatic islet or β -cell function.

Typically, an agent is selected if it changes the expression of a differentially expressed protein towards that of a subject having more normal pancreatic islet or β -cell function.

In a further aspect, the present invention provides a method for the identification of an agent or agents for use in the treatment of pancreatic islet or pancreatic β -cell disorders comprising the steps of:

(a) identification of experimental and/or clinical paradigms that exhibit differential levels of pancreatic islet or pancreatic β -cell dysfunction;

(b) identification of differentially expressed proteins in tissues of animals or humans exhibiting differential levels of islet or β -cell dysfunction;

(c) selecting an agent that converts the expression of differentially expressed proteins in islet or β -cell dysfunction state to the normal state for use in the treatment of the islet or β -cell dysfunction states.

In a further aspect, the present invention provides a method of making a pharmaceutical composition which comprises having identified an agent using the above method, the further step of manufacturing the agent and formulating it with an acceptable carrier to provide the pharmaceutical composition.

In a further aspect, the present invention provides the use of an agent identified by the above method for the preparation of a medicament for the treatment of a condition characterised by islet or β -cell dysfunction. These conditions include non-insulin dependent diabetes (type 2 diabetes), syndrome X or insulin resistance syndrome or gestational diabetes.

In a further aspect, the present invention provides a method of treating a condition characterised by islet or β -cell dysfunction in a patient, the method comprising administering a therapeutically or prophylactically effective amount of such an agent identified by the above method.

In a further aspect, the present invention provides a method of determining the nature or degree of pancreatic islet or β -cell dysfunction in a human or animal subject, the method comprising:

(a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of pancreatic islet or β -cell function;

(b) obtaining a sample of the tissue from the subject;

(c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the sample; and

(d) relating the determination to the nature or degree of the pancreatic islet or β -cell function by reference to a previous correlation between such a determination and clinical information.

Conveniently, the patient sample used in method can be a tissue sample or body fluid sample or urine. This method allows the type of pancreatic islet or β -cell dysfunction of a patient to be correlated to different types to prophylactic or therapeutic treatment available in the art, thereby enhancing the likely response of the patient to the therapy.

In a further aspect, the present invention provides a method of treatment by the use of an agent that will restore the expression of one or more differentially expressed proteins in the pancreatic islet or β -cell dysfunction state to that found in the normal state in order to prevent the development of non-insulin dependent diabetes in a pre-diabetic subject.

In a further aspect, the present invention provides a method whereby the pattern of differentially expressed proteins in a tissue sample or body fluid sample or urine of an individual with pancreatic islet or β -cell dysfunction is used to predict the most appropriate and effective therapy to alleviate the pancreatic islet or β -cell dysfunction state and to monitor the success of that treatment.

In a further aspect, the present invention provides a protein which is differentially expressed in relevant tissue from, or representative of subjects having differential levels of pancreatic islet or β -cell dysfunction and which is as obtainable by the method of two-dimensional gel electrophoresis carried out on said tissue or a protein-containing extract thereof, the method comprising:

(a) providing non-linear immobilized pH gradient (IPG) strips of acrylamide polymer 3 mm x 180 mm;

(b) rehydrating the IPG strips in a cassette containing 25 ml. of an aqueous solution of urea (8M), 3-
5 [(cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of acids and bases of pH 3.5 to 10 (2% w/v) and a trace of Bromophenol Blue;

(c) emptying the cassette of liquid, transferring
10 the strips to an electrophoretic tray fitted with humid electrode wicks, electrodes and sample cups, covering the strips and cups with low viscosity paraffin oil;

(d) applying 200 micrograms of an aqueous solution of dried, powdered material of the relevant body tissue
15 in urea (8M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM), SDS (0.05% w/v) and a trace of Bromophenol Blue to the sample cups, at the cathodic end of the IPG strips;

(e) carrying out isoelectric focusing on the gel at a voltage which increases linearly from 300 to 3500 V
20 during 3 hours, followed by another 3 hours at 3500 V, and thereafter at 5000V for a time effective to enable the proteins to migrate in the strips to their pI-dependent final positions;

(f) equilibrating the strips within the tray with
25 100 ml of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 minutes;

(g) replacing this solution by 100 ml. of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea
30 (6M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of Bromophenol Blue for 5 minutes;

(h) providing a vertical gradient slab gel 160 x

200 x 1.5 mm of acrylamide/piperazine-diacrylyl cross-linker(9-16%T/2.6%C), polymerised in the presence of TEMED (0.5% w/v), ammonium persulphate (0.1% w/v) and sodium thiosulphate (5 mM), in Tris-HCl (0.375M) pH 8.8 as leading buffer;

(i) over-layering the gel with sec-butanol for about 2 hours, removing the overlay and replacing it with water;

(j) cutting the IPG gel strips to a size suitable for the second dimensional electrophoresis, removing 6 mm from the anode end and 14 mm from the cathode end;

(k) over-layering the slab gel with an aqueous solution of agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1% w/v) as leading buffer, heated to 70°C and loading the IPG gel strips onto the slab gel through this over-layered solution;

(l) running the second dimensional electrophoresis at a constant current of 40 mA at 8-12°C for 5 hours; and
(m) washing the gel.

Examples of differentially expressed protein described herein and found in samples from pancreatic islet or β -cells include POM1, POM2, POM3, POM4, POM6, POM6, POM7, POM8, POM9, POM10, POM11, POM12, POM13, POMT1, POMT2, POMT3, POMT4, POMT5, POMT5, POMT11, POMT12, POMT13, PSEM14 AND PSEM15.

Alternatively, fingerprint proteins may be used in methods for identifying compounds useful for the treatment of pancreatic islet and/or β -cell dysfunction. 'Target protein', as used herein, refers to a differentially expressed protein involved in pancreatic islet and/or β -cell function such that modulation of the

expression of that protein may act to prevent or ameliorate pancreatic islet and/or β -cell disorders including, but not limited to, non-insulin dependent diabetes.

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This invention is based, in part, on systematic search strategies involving modulations of pancreatic islet and/or β -cell structure and function and non-insulin dependent diabetes experimental paradigms, coupled with sensitive detection of proteins by 2D-electrophoresis.

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The invention further provides methods for the identification of compounds that modulate the expression of proteins involved in pancreatic islet and/or β -cell mass and pancreatic islet and/or β -cell function and processes relevant to the regulation of pancreatic insulin secretion. Still further, the present invention describes methods for the prevention and/or treatment of non-insulin dependent diabetes, which may involve the administration of such compounds to individuals predisposed to or exhibiting pancreatic islet and/or β -cell dysfunction. These individuals include, but are not limited to, humans and animals with non-insulin dependent diabetes. These individuals also include pregnant humans or animals that are at risk of producing offspring with a low birthweight and low pancreatic β -cell mass.

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Additionally, the present invention describes methods for prognostic and diagnostic evaluation of various pancreatic islet and/or β -cell disorders and for the identification of subjects exhibiting a predisposition to such disorders.

The examples presented below demonstrate the successful use of the experimental paradigms of the invention to identify target proteins associated with a reduced pancreatic islet and/or β -cell mass and proteins associated with pancreatic islet and/or β -cell dysfunction.

Definitions

"Differential expression", as used herein, refers to at least one recognisable difference in tissue protein expression. It may be a quantitatively measurable, semi-quantitatively estimatable or qualitatively detectable difference in tissue protein expression. Thus, a differentially expressed protein (herein DEP) may be strongly expressed in tissue in the normal state and less strongly expressed or not expressed at all in tissue in the pancreatic islet or β -cell dysfunctional state. Conversely, it may be strongly expressed in tissue in the disorder state and less strongly expressed or not expressed at all in the normal state. Similarly, the differential expression can be either way around in the comparison between untreated and treated tissue. Further, expression may be regarded as differential if the protein undergoes any recognisable change between the two states under comparison.

The term "paradigm" means a prototype example, test model or standard.

Wherever a differentially expressible protein is used in the screening procedure, it follows that there must have been at some time in the past a preliminary step of establishing a paradigm by which the differential

expressibility of the protein was pre-determined. Once the paradigm has been established, it need not be re-established on every occasion that a screening procedure is carried out. The term "establishing a paradigm" is to be construed accordingly.

"Islet or β -cell dysfunction" includes conditions in which the mass of these cell types is reduced in patients and/or where the cells have reduced function as compared to normal cells, e.g. in the production of insulin. Conditions characterised by islet or β -cell dysfunction include non-insulin dependent diabetes or type 2 diabetes, syndrome X or insulin resistance syndrome or gestational diabetes.

"Relevant tissue" means any tissue which undergoes a biological change in response to the action of insulin in the body, or any other tissue affected by this change.

"Tissue... ..representative of... ..subjects" means any tissue in which the above-mentioned biological change can be simulated for laboratory purposes and includes, for example, a primary cell culture or cell line derived ultimately from relevant tissue.

The term "subjects" includes human and animal subjects.

The treatments referred to above can comprise the administration of one or more drugs or foodstuffs, and/or other factors such as diet or exercise.

The differentially expressed proteins (DEPs) include "fingerprint proteins", "target proteins" or "pathway

proteins".

The term "fingerprint protein", as used herein, means a DEP, the expression of which can be used, alone or together with other DEPs, to monitor or assess the condition of a patient suspected of suffering from pancreatic islet or β -cell dysfunction. Since these proteins will normally be used in combination, especially a combination of four or more, they are conveniently termed "fingerprint proteins", without prejudice to the possibility that on occasions they may be used singly or along with only one or two other proteins for this purpose. Such a fingerprint protein or proteins can be used, for example, to diagnose a particular type of pancreatic islet or β -cell dysfunction and thence to suggest a specific treatment for it.

The term "diagnosis", as used herein, includes the provision of any information concerning the existence, non-existence or probability of the disorder in a patient. It further includes the provision of information concerning the type or classification of the disorder or of symptoms which are or may be experienced in connection with it. It encompasses prognosis of the medical course of the disorder.

The term "target protein", as used herein, means a DEP, the level or activity of which can be modulated by treatment to alleviate an disorder characterised by pancreatic islet or β -cell dysfunction. Modulation of the level or activity of the target protein in a patient may be achieved, for example, by administering the target protein, another protein or gene which interacts with it

or an agent which counteracts or reduces it, for example an antibody to the protein, competitive inhibitor of the protein or an agent which acts in the process of transcription or translation of the corresponding gene.

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The term "alleviate", as used herein, in relation to pancreatic islet or β -cell dysfunction means any form of reducing one or more undesired symptoms or effects thereof. Any amelioration of the pancreatic islet or β -cell dysfunction of the patient falls within the term "alleviation".

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Alternatively or additionally, the DEPs can interact with at least one other protein or with a gene involved in the regulation of pancreatic islet or β -cell function. Such other proteins are termed herein "pathway proteins" (PPs). The term is applied to the protein with which it the DEP interacts, not to the DEP itself, although a pathway protein can be another DEP.)

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By way of example, embodiments of the present invention will now be described in more detail with reference to the accompanying figures.

25 Brief Description of the Figures

Figure 1 shows a computer images of stained 2-DGE gels from pancreatic islet cells of lean control mice, identifying spots thereon, including DEPs.

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Figures 2 to 7 and Figures 9 to 13 show the comparative expression of the DEPs together with quantification of the differential expression.

Figures 8 and 14 show a control protein (C1230) that is not differentially expressed.

Detailed Description

5 Methods and compositions for the treatment of pancreatic islet and/or β -cell disorders including, but not limited to, non-insulin dependent diabetes. Proteins termed 'target proteins' and/or fingerprint proteins are described which are differentially expressed in
10 pancreatic islet and/or β -cell disorders states relative to their expression in normal states and/or which are differentially expressed in response to manipulations relevant to the regulation of pancreatic islet and/or β -cell mass and/or function. Additionally, proteins termed
15 'pathway proteins' are described which interact with proteins involved in pancreatic islet and/or β -cell mass and/or function regulation. Methods for the identification of such fingerprint target and pathway proteins are also described.

20 Described below are methods for the identification of compounds, which modulate the expression of proteins, involved in pancreatic islet and/or β -cell mass and/or function regulation. Additionally described below are
25 methods for the treatment of pancreatic islet and/or β -cell disorder states including, but not limited to, non-insulin dependent diabetes.

Also discussed below are methods for prognostic and
30 diagnostic evaluation of pancreatic islet and/or β -cell disorder states and for the identification of subjects exhibiting a predisposition to such disorders.

1 Identification of differentially expressed and
pathway proteins

In one embodiment, the present invention concerns methods for the identification of proteins which are involved in pancreatic islet and/or β -cell disorders and/or which are involved in non-insulin dependent diabetes. Such proteins may represent proteins, which are differentially expressed in pancreatic islet and/or β -cell disorder states relative to their expression in normal states.

Further, such proteins may represent proteins that are differentially expressed or regulated in response to manipulation relevant to increasing pancreatic islet and/or β -cell mass or altering function. Such differentially expressed proteins may represent 'target' or 'fingerprint' proteins. Methods for the identification of such proteins are described in Section 1.1. Methods for the further characterisation of such differentially expressed proteins and for their identification as target and/or fingerprint proteins are presented below in Section 1.3.

In addition, methods are described herein in Section 1.3, for the identification of proteins termed pathway proteins involved in pancreatic islet and/or β -cell disorder states and/or non-insulin dependent diabetes. Pathway proteins, as used herein, refer to a protein, which exhibits the ability to interact with other proteins relevant to pancreatic islet and/or β -cell disorder states. A pathway protein may be differentially expressed and therefore may have the characteristics of a target or fingerprint protein.

'Differential expression', as used herein, refers to both

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qualitative as well as quantitative differences in
protein expression. Thus a differentially expressed
protein may qualitatively have its expression activated
or completely inactivated in normal versus pancreatic
5 islet and/or β -cell disorder state or under control
versus experimental conditions. Such a qualitatively
regulated protein will exhibit an expression pattern
within a given tissue or cell type, which is detectable
in either control or pancreatic islet and/or β -cell
10 disorder subject, but not detectable in both.

Alternatively, such a qualitatively regulated protein
will exhibit an expression pattern within one or more
cell types in the pancreatic islet, which is detectable
in either control or experimental subjects but not
15 detectable in both. 'Detectable', as used herein, refers
to a protein expression pattern, which are detectable
using techniques such as differential display 2D
electrophoresis.

20 Alternatively, a differentially expressed protein may
have its expression modulated, i.e. quantitatively
increased or decreased, in normal versus pancreatic islet
and/or β -cell disorder states or under control versus
experimental conditions. The degree to which expression
25 differs in normal versus pancreatic islet and/or β -cell
disorder states or control versus experimental states
need only be large enough to be visualised via standard
characterisation techniques, such as silver staining of
2D-electrophoretic gels. Other such standard
30 characterisation techniques by which expression
differences may be visualised are well known to those
skilled in the art. These include successive
chromatographic separations of fractions and comparisons

of the peaks, capillary electrophoresis and separations using micro-channel networks, including on a micro-chip.

Chromatographic separations can be carried out by high performance liquid chromatography as described in Pharmacia literature, the chromatogram being obtained in the form of a plot of absorbance of light at 280 nm against time of separation. The material giving incompletely resolved peaks is then re-chromatographed and so on.

Capillary electrophoresis is a technique described in many publications, for example in the literature "Total CE Solutions" supplied by Beckman with their P/ACE 5000 system. The technique depends on applying an electric potential across the sample contained in a small capillary tube. The tube has a charged surface, such as negatively charged silicate glass. Oppositely charged ions (in this instance, positive ions) are attracted to the surface and then migrate to the appropriate electrode of the same polarity as the surface (in this instance, the cathode). In this electroosmotic flow (EOF) of the sample, the positive ions move fastest, followed by uncharged material and negatively charged ions. Thus, proteins are separated essentially according to charge on them.

Micro-channel networks function somewhat like capillaries and can be formed by photoablation of a polymeric material. In this technique, a UV laser is used to generate high energy light pulses that are fired in bursts onto polymers having suitable UV absorption characteristics, for example polyethylene terephthalate

or polycarbonate. The incident photons break chemical bonds with a confined space, leading to a rise in internal pressure, mini-explosions and ejection of the ablated material, leaving behind voids which form micro-channels. The micro-channel material achieves a separation based on EOF, as for capillary electrophoresis. It is adaptable to micro-chip form, each chip having its own sample injector, separation column and electrochemical detector: see J.S.Rossier et al., 1999, Electrophoresis 20: pages 727-731.

Differentially expressed proteins may be further described as target proteins and/or fingerprint proteins. 'Fingerprint proteins', as used herein, refer to a differentially expressed protein-whose expression pattern may be utilised as part of a prognostic or diagnostic pancreatic islet and/or β -cell disorder evaluation or which, alternatively, may be used in methods for identifying compounds useful for the treatment of pancreatic islet and/or β -cell disorder states. A fingerprint protein may also have characteristics of a target protein or a pathway protein.

'Target protein', as used herein, refers to a differentially expressed protein involved in pancreatic islet and/or β -cell disorder states and/or non-insulin dependent diabetes such that modulation of the level or activity of the protein may act to prevent the development on pancreatic islet and/or β -cell disorder states including, but not limited to, non-insulin dependent diabetes. A target protein may also have the characteristics of a fingerprint protein or a pathway protein.

1.1 Method for the identification of differentially expressed proteins

A variety of methods may be used for the identification of proteins, which are involved in pancreatic islet and/or β -cell disorder states and/or which may be involved in non-insulin dependent diabetes. Described in Section 1.1.1 are several experimental paradigms, which may be utilised for the generation of subjects, and samples, which may be used for the identification of such proteins. Material from the paradigm control and experimental subjects may be characterised for the presence of differentially expressed protein sequences as discussed below in Section 1.1.2.

1.1.1 Paradigms for the identification of differentially expressed proteins

Among the paradigms that may be utilised for the identification of differentially expressed proteins involved in pancreatic islet and/or β -cell disorder states are paradigms designed to analyse those proteins that are differentially expressed between normal and pancreatic islet and/or β -cell disorder states including, but not limited to, non-insulin dependent diabetes, gestational diabetes and impaired glucose tolerance.

In one embodiment of such a paradigm, pancreatic islet tissue from normal and pancreatic islet and/or β -cell disorder subjects would be compared. Such subjects could include, but would not be limited to, subjects with non-insulin dependent diabetes, impaired glucose tolerance, gestational diabetes, first degree relatives of non-insulin dependent diabetes. It could also involve a comparison of normal and pregnant individuals or normal

subjects and subjects who have resisted the development of pancreatic islet and/or β -cell disorders despite the presence of insulin resistance. Appropriate tissues would include, but not be limited to, blood and pancreatic islets.

Among additional paradigms would include a comparison of non-insulin diabetic subjects and subjects whose pancreatic islet and/or β -cell function had been improved by, but not limited to, dietary restriction or modification, insulin sensitiser drugs, such as troglitazone and rosiglitazone, metformin, exercise and β_3 -adrenoceptor agonists such as BRL 35135 and 26830.

In a further paradigm, which may be utilised for the identification of differentially expressed proteins involved in pancreatic islet and/or β -cell disorder states are paradigms designed to analyse those proteins which may be involved in genetic models of pancreatic islet and/or dysfunction or alterations in β -cell mass and/or non-insulin dependent diabetes. Accordingly, such paradigms are referred to as 'genetic pancreatic islet and/or β -cell paradigms'. In the case of mice, for example, such paradigms may identify the proteins regulated either directly or indirectly by the ob/ob, db/db, tub or fat gene products. In rats, such a paradigm may identify proteins regulated either directly or indirectly by the fa gene product. Such paradigms may also identify the proteins regulated by the genetic background and which result in the diabetic condition when the ob/ob, db/db and fa gene products are expressed on the diabetes sensitive background

In one embodiment of such a paradigm, test subjects may include ob/ob, db/db, tub/tub or fat/fat experimental mice and lean littermate controls on both the C57BI/6 and C57BI/Ks backgrounds. Test subjects could also include fa/fa and male and female ZDF rats. Samples of pancreas would be obtained and islets prepared free of exocrine pancreas. The examples given in Sections and below demonstrate the use of such genetic paradigms in identifying proteins which are differentially expressed in pancreatic islet and/or β -cell disorder animals versus normal animals.

In additional embodiments, ob/ob, db/db, tub/tub and/or fat/fat mice and/or fa/fa and ZDF rats and lean control animals may be treated with drugs that improve pancreatic islet and/or β -cell function. Such drugs include, but are not limited to, insulin sensitisers such as the thiazolidinediones, rosiglitazone, pioglitazone or troglitazone, the oxazolidinones JTT501, non-thiazolidinedione PPAR gamma activators, RXR activators that form heterodimers with PPAR gamma, metformin and β_3 -adrenoceptor agonists. Such a paradigm allows the identification of target proteins.

In a further additional embodiment, ob/ob, db/db, tub/tub and/or fat/fat mice and/or fa/fa and/or ZDF rats and lean controls may be offered dietary treatments to either worsen the insulin resistant state or improve insulin sensitivity. For example, either lean or insulin resistant animals could be provided with a high fat diet to exacerbate the insulin resistant state.

In one embodiment of such a paradigm, C57BI/6 and

C57BI/Ks wild-type mice would be fed on a high fat diet or a cafeteria diet consisting of human snack foods. The C56BI/Ks mice have a smaller pancreatic islet cell mass than the C57BI/6 mice. Hence when demand for insulin secretion is increased by the development of insulin resistance by feeding on a high fat or cafeteria diet, the C57BI/Ks mice are compromised more. Thus, this paradigm can be used to select proteins that are associated with a predisposition to a pancreatic islet and/or β -cell disorder state. The paradigm can be further refined by incorporating drug treatment paradigms.

Some native animal strains do not exhibit either pancreatic islet and/or β -cell dysfunction or non-insulin dependent diabetes in the wild but do when fed a laboratory chow or other laboratory diets. These include the desert rodents, the spiny mouse and the sand rat. Comparison of animals fed on the natural diet and those fed on a laboratory diet allows identification of proteins associated with pancreatic islet and/or β -cell disorders.

Further paradigms that may be utilised for the identification of differentially expressed protein involved in pancreatic islet and/or β -cell disorder states may include paradigms in which pregnant mice or rats may be fed on a low protein diet (typically 6% fat) during pregnancy and/or lactation whereas control animals are fed on a diet containing a normal protein content (typically 15%). After weaning, offspring may be fed on a high fat diet. Such latter offspring develop non-insulin dependent diabetes. Comparison between these

various animals fed on different diets during gestation, weaning and/or in adult life allows the identification of differentially expressed proteins associated with pancreatic islet and/or β -cell dysfunction including, but not limited to, changes that predispose to the development of non-insulin dependent diabetes.

Paradigms that involve systems in which the pancreatic islet or β -cell mass is manipulated may also be utilised. Such paradigms include pregnant animals and animals treated with agents that are known to produce an increase in pancreatic islet β -cell mass or proliferation of β -cells. Such agents include prolactin.

In addition to whole animal studies, paradigms include systems in which isolated pancreatic islets or β -cells are incubated in vitro with agents that stimulate β -cell proliferation. Furthermore, the paradigm systems in which proliferation of β -cell lines such as Rin m5F, Rin 5F, HIT 15, MIN and BRINS cells is initiated in vitro.

1.1.2 Analysis of paradigm material

In order to identify differentially expressed proteins, pancreatic islets and/or pancreatic β -cell from subjects utilised in paradigms such as those described above in 1.1.1 are obtained. Usually this will involve digestion of pancreatic tissue with enzymes such as collagenase. Methods for the isolation of pancreatic cells are well known to those in the art, as are methods of obtaining isolated β -cells from the islet tissue by such means as FACS sorting. In addition, blood and body fluids may be analysed since the differentially expressed proteins in pancreatic islets or β -cells might be released into the

circulations.

Whole pancreatic islets, β -cell, and other islet cells may be used, as may whole pancreas including the exocrine tissue. Sub-cellular fractions of islets and isolated cells might also be used. Particularly useful sub-cellular fractions include the nuclear protein fraction.

1.2 Methods for the identification of pathway proteins

Methods are described herein for the identification of pathway proteins. 'Pathway protein', as used herein, refers to a protein which exhibits the ability to interact with differentially expressed proteins involved in pancreatic islet and/or β -cell disorders and/or to interact with differentially expressed proteins which are relevant to non-insulin dependent diabetes. A pathway protein may be differentially expressed and, therefore, may have the characteristics of a target and/or fingerprint protein.

Any method suitable for detecting protein-protein interactions may be employed for identifying pathway proteins by identifying interactions between candidate proteins and proteins known to be differentially expressed in pancreatic islet and/or β -cell disorder states and/or non-insulin dependent diabetes regulation. Such differentially expressed proteins may be cellular or extracellular proteins. Those proteins, which interact with such differentially expressed proteins, represent pathway gene products.

Among the traditional methods, which may be employed, are co-immunoprecipitation, crosslinking and co-purification

through gradients or chromatographic columns. Utilising procedures such as these allows for the identification of pathway proteins. Once identified, a pathway protein may be used, in conjunction with standard techniques, to

5 identify its corresponding pathway gene. For example, at least a portion of the amino acid sequence of the pathway gene product may be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, e.g. Creighton (1983)

10 'Proteins: Structures and Molecular Principles', W.H. Freeman & Co., N.Y., pp. 34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for pathway gene sequences. Screening may be accomplished, 15 for example, by standard hybridisation or PCR techniques. Techniques for the generation of oligonucleotide mixtures and the screening are well-known (see, e.g. Ausubel, supra. and PCR Protocols: A Guide to Methods and Applications (1990) Innis, M. et al., eds. Academic Press 20 Inc., New York).

One method, which detects protein interactions in vivo, the two-hybrid system, is described in detail for illustration only and not by way of limitation. One 25 version of this system has been described (Chien et al (1991) Proc. Natl. Acad. Sci. USA, 88, 9578-9582) and is commercially available from Clontech (Palo Alto, Calif.).

Briefly, utilising such a system, plasmids are 30 constructed that encode two hybrid proteins: one consists of the DNA-binding domain of a transcription activator protein fused to a known protein, in this case, the differentially expressed protein known to be involved

in pancreatic islet and/or β -cell disorder states and/or non-insulin dependent diabetes regulation, and the other consists of the transcription activator protein's activation domain fused to an unknown protein that is encoded by a cDNA which has been recombined into this plasmid as part of a cDNA library. The plasmids are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (e.g. lacZ) whose regulatory region contains the transcription activator's binding sites. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the activation domain hybrid cannot because it cannot localise to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with a known differentially expressed 'bait' protein. Total genomic or cDNA sequences are translationally fused to the DNA encoding an activation domain, e.g. an activated domain of GAL-4. This library and a plasmid encoding a hybrid of the bait protein product fused to the DNA-binding domain are co-transformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. By way of example rather than limitation, the bait gene can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These

colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

5

A cDNA library of the cell line from which proteins that interact with bait proteins are to be detected can be made using methods routinely practised in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the activation domain of GAL4. This library can be co-transformed along with the bait-gene GAL4 fusion plasmid into a yeast strain, which contains a lacZ gene driven by a promoter which contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 activation domain, that interacts with bait gene product will reconstitute an active GAL4 protein and thereby drive expression of the lacZ gene. Colonies which express lacZ can be detected by their blue color in the presence of X-gal. The cDNA can then be purified from these strains, and used to produce and isolate the bait gene-interacting protein using techniques routinely practised in the art.

25 Once a pathway protein has been identified and isolated, it may be further characterised as, for example, discussed below, in Section 1.3.

1.3 Characterisation of differentially expressed and pathway proteins

30

Differentially expressed proteins, such as those identified via the methods discussed above in Section 1.1, and pathway genes, such as those identified via the

methods discussed above in Section 1.2, as well as genes identified by alternative means, may be further characterised by utilising, for example, methods such as those discussed herein. Such proteins will be referred to herein as 'identified proteins'.

Analyses such as those described herein, yield information regarding the biological function of the identified proteins. An assessment of the biological function of the differentially expressed proteins, in addition, will allow for their designation as target and/or fingerprint proteins.

Specifically, any of the differentially expressed proteins whose further characterisation indicates that a modulation of the proteins expressed or a modulation of the proteins activity may ameliorate any of the pancreatic islet and/or β -cell disorders will be designated 'target proteins', as defined above, in Section 1. Such target proteins, along with those discussed below, will constitute the focus of the compound discovery strategies discussed below in Section 3. Further, such target proteins and/or modulating compounds can be used as part of the treatment and/or prevention of pancreatic islet and/or β -cell disorders and/or non-insulin dependent diabetes.

Any of the differentially expressed proteins whose further characterisation indicates that such modulations may not positively affect pancreatic islet and/or β -cell disorders, but whose expression pattern contributes to a protein 'fingerprint' pattern correlative of, for example, a pancreatic islet and/or β -cell disorder state,

will be designated a 'fingerprint protein'. 'Fingerprint patterns' will be more fully discussed below, in Section 7.1. It should be noted that each of the target proteins may also function as fingerprint proteins, as well as may all or a portion of the pathway proteins.

It should further be noted that the pathway proteins may also be characterised according to techniques such as those described herein. Those pathway proteins which yield information indicating that they are differentially expressed and that modulation of the proteins expression or a modulation of the proteins expression or a modulation of the proteins activity may ameliorate any of the pancreatic islet and/or β -cell disorders of interest will also be designated 'target proteins'. Such target proteins, along with those discussed above, will constitute the focus of the compound discovery strategies discussed below, in Section 3 and can be used as part of the treatment methods described in Section 4 below.

It should be additionally noted that the characterisation of one or more of the pathway proteins may reveal a lack of differential expression, but evidence that modulation of the gene's activity or expression may, nonetheless, ameliorate pancreatic islet and/or β -cell disorder symptoms. In such cases, these genes and gene products would also be considered a focus of the compound discovery strategies of Section 3 below.

In instances wherein a pathway proteins characterisation indicates that modulation of gene expression or gene product activity may not positively affect pancreatic islet and/or β -cell disorders of interest, but whose

expression is differentially expressed and contributes to a gene expression fingerprint pattern correlative of, for example, a pancreatic islet and/or β -cell disorder state, such pathway genes may additionally be designated as fingerprint genes.

A variety of techniques can be utilised to further characterise the identified proteins. First, the corresponding nucleotide sequence of the identified protein may be obtained by utilising standard techniques well known to those of skill in the art, may, for example, be used to reveal homologies to one or more known sequence motifs which may yield information regarding the biological function of the identified protein.

Secondly, the biological function of the identified proteins may be more directly assessed by utilising relevant in vivo and in vitro systems. In vivo systems may include, but are not limited to, animal systems which naturally exhibit pancreatic islet and/or β -cell disorder-like symptoms, or ones which have been engineered to exhibit such symptoms. Further, such systems may include systems for the further characterisation of pancreatic islet and/or β -cell disorders, and/or non-insulin dependent diabetes, and may include, but are not limited to, naturally occurring and transgenic animal systems such as those described above, in Section 1.1.1, and Section 2.2.1 below. In vitro systems may include, but are not limited to, cell-based systems comprising cell types known to produce and secrete insulin. Such cells may be wild type cells, or may be non-wild type cells containing modifications known

to, or suspected of, contributing to a pancreatic islet and/or β -cell disorder of interest. Such systems are discussed in detail below, in Section 2.2.2.

5 In further characterising the biological function of the identified proteins, the expression of these proteins may be modulated within the in vivo and/or in vitro systems, i.e. either overexpressed or underexpressed in, for example, transgenic animals and/or cell lines, and
10 its subsequent effect on the system then assayed. Alternatively, the activity of the identified protein may be modulated by either increasing or decreasing the level of activity in the in vivo and/or in vitro system of interest, and its subsequent effect then assayed.

15 The information obtained through such characterisations may suggest relevant methods for the treatment of pancreatic islet and/or β -cell disorders involving the protein of interest. Further, relevant methods for the control of non-insulin dependent diabetes involving the
20 protein of interest may be suggested by information obtained from such characterisations. For example, treatment may include a modulation of protein expression and/or protein activity. Characterisation procedures
25 such as those described herein may indicate where such modulation should involve an increase or a decrease in the expression or activity of the protein of interest. Such methods of treatment are discussed below in Section 4.

30 2 Differentially expressed and pathway proteins

Identified proteins, which include, but are not limited to, differentially expressed proteins such as those

identified in Section 1.1 above, and pathway proteins, such as those identified in Section 1.2 above, are described herein. Specifically, the amino acid sequences of such identified proteins are described. Further, antibodies directed against the identified protein, and cell- and animal-based models by which the identified proteins may be further characterised and utilised are also discussed in this Section.

2.1 Antibodies specific for differentially expressed or pathway proteins

The present invention also relates to methods for the production of antibodies capable of specifically recognising one or more differentially expressed or pathway protein epitopes. Such antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanised or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be utilised as part of pancreatic islet and/or β -cell treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of fingerprint, target, or pathway gene proteins, or for the presence of abnormal forms of such proteins.

For the production of antibodies to a differentially expressed or pathway protein, various host animals may be immunised by injection with a differentially expressed or pathway protein, or a portion thereof. Such host animals may include, but are not limited to, rabbits, mice and

rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including active substances such as lysolecithin, Pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvant such as BCG bacille Calmette-Fuerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunised with an antigen, such as target proteins, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunised by injection with differentially expressed or pathway protein supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique, which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein (1975, *Nature* 256; 495-497; and US Patent No: 4,376,110), the human β -cell hybridoma technique (Kosbor, et al., 1983, *Immunology Today* 4: 72; Cole, et al., 1983, *Proc. Natl. Acad. Sci. USA* 80; 2026-2030), and the EBV-hybridoma technique (Cole, et al., 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in

vivo makes this the presently preferred method of production.

5 In addition, techniques developed for the production of
'chimeric antibodies' (Morrison, et al., 1984, Proc.
Natl. Acad. Sci. 81: 6851-6855; Neuberger, et al., 1984,
Nature 312: 604-608; Takeda, et al., 1985, Nature 314:
10 452-454) by splicing the genes from a mouse antibody
molecule of appropriate antigen specificity together with
genes from a human antibody molecule of appropriate
biological activity can be used. A chimeric antibody is
a molecule in which different portions are derived from
different animal species, such as those having a variable
region derived from a murine mAb and a human
15 immunoglobulin constant region.

Alternatively, techniques described for the production of
single chain antibodies (US Patent No: 4,946,778; Bird,
1988, Science 242: 423-426; Huston, et al., 1988, Proc.
20 Natl. Acad. Sci. USA 85: 5879-5883; and Ward, et al.,
1989, Nature 334: 544-546) can be adapted to produce
differentially expressed or pathway protein-single chain
antibodies. Single chain antibodies are formed by
linking the heavy and light chain fragments of the Fv
25 region via an amino acid bridge, resulting in a single
chain polypeptide.

Antibody fragments, which recognise specific epitopes,
may be generated by known techniques. For example, such
30 fragments include, but are not limited to, the F(ab')₂
fragments which can be produced by pepsin digestion of
the antibody molecule and the Fab fragments which can be
generated by reducing the disulfide bridges of the F(ab')₂

fragments. Alternative, Fab expression libraries may be constructed (Huse, et al., 1989, Science 246: 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

5

2.2 Cell- and animal-based model systems

Described herein are cell- and animal- based systems, which act as models for pancreatic islet and/or β -cell disorders. These systems may be used in a variety of applications. For example, the animal-based model systems can be utilised to identify differentially expressed proteins via one of the paradigms described above, in Section 1.1.1. Cell- and animal-based model systems may be used to further characterise differentially expressed and pathway proteins, as described above in Section 1.3. Such further characterisation may, for example, indicate that a differentially expressed protein is a target protein. Second, such assays may be utilised as part of screening strategies designed to identify compounds which are capable of ameliorating pancreatic islet and/or β -cell disorder symptoms, as described below. Thus, the animal- and cell-based models may be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in treating such pancreatic islet and/or β -cell disorders. In addition, as described in detail below, in Section 6, such animal models may be used to determine the LD₅₀ and the ED₅₀ in animal subjects, and such data can be used to determine the in vivo efficacy of potential pancreatic islet and/or β -cell disorder treatments, including treatments for non-insulin diabetes.

2.2.1 Animal-based systems

Animal-based model systems of pancreatic islet and/or β -cell disorders may include, but are not limited to, non-recombinant and engineered transgenic animals.

5

Non-recombinant animal models for pancreatic islet and/or β -cell disorders may include, for example, genetic models. Such genetic pancreatic islet and/or β -cell models may include, for example, mouse models of non-insulin dependent diabetes and/or obesity such as mice homozygous for the autosomal recessive ob, db, or tub alleles. It could also include rat models, for example ZDF rats.

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Non-recombinant, non-genetic animal models of pancreatic islet and/or β -cell disorder may include, for example, rats or mice fed on a diet containing a large amount of fat. Such diets could be synthetic diets in which the fat content (by calorific value) is more than 50%.

20

Alternative human foods with a high fat content, such as salami and butter, may be provided to the animals. This model is particularly useful if it utilises low birth weight pups arising from a pregnant female fed on a low protein diet.

25

Additionally, animal models exhibiting pancreatic islet and/or β -cell disorder-like symptoms may be engineered by utilising, for example, the gene sequences of target proteins such as those described above in Section 2, in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the

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animal of interest, or, if endogenous gene sequences of target proteins are present, they may either be overexpressed or, alternatively, may be disrupted in order to underexpress or inactivate gene expression of target proteins.

In order to overexpress the target gene sequence of a target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the animal and cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilised in the absence of undue experimentation.

For underexpression of an endogenous gene sequence of a target protein, such a sequence may be isolated and engineered such that when reintroduced into the genome of the animal of interest, the endogenous gene alleles of the target protein will be inactivated. Preferably, the engineered gene sequence of the target protein is introduced via gene targeting such that the endogenous sequence is disrupted upon integration of the engineered target gene sequence into the animal's genome.

Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, mini-pigs, goats and non-human primates, e.g. baboons, squirrels, monkeys and chimpanzees may be used to generate pancreatic islet and/or β -cell disorder animal models.

Any technique known in the art may be used to introduce a target gene transgene of a target protein into animals to produce the founder lines of transgenic animals. Such

techniques include, but are not limited to, pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, US Pat.No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82: 6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56: 313-321); electroporation of embryos (Lo, 1983, Mol. Cell Biol. 3: 1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57: 717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115: 171-229.

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4: 761-763). The transgene may be integrated as a single transgene or in concatamers, e.g. head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236). The regulatory sequences required to such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the target gene transgene be integrated into the chromosomal site of the endogenous target gene, gene targeting is preferred. Briefly, when such a technique is to be utilised, vectors containing some nucleotide sequences homologous to the gene of the

endogenous target protein of interest are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of, the nucleotide sequence of the endogenous target gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene of interest in only that cell type, by following, for example, the teaching of Gu et al. (Gu, H. et al., 1994, Science 265: 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant target gene and protein may be assayed utilising standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyse animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridisation analysis, and RT-PCR. Samples of target protein-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the transgene protein of interest.

The target protein transgenic animals that express target gene mRNA or target protein transgene peptide (detected immunocytochemically, using antibodies directed against target protein epitopes) at easily detectable levels

should then be further evaluated to identify those animals which display characteristic pancreatic islet and/or β -cell disorder-like symptoms. Such symptoms may include, for example, obesity, glucose intolerance, hyperinsulinaemia, glycosuria and/or non-insulin dependent diabetes. Additionally, specific cell types within the transgenic animals may be analysed and assayed for cellular phenotypes characteristic of pancreatic islet and/or β -cell disorders. Further, such cellular phenotypes may include an assessment of a particular cell types fingerprint pattern of expression and its comparison to known fingerprint expression profiles of the particular cell type in animals exhibiting pancreatic islet and/or β -cell disorders. Such transgenic animals serve as suitable model systems for pancreatic islet and/or β -cell disorders.

Once target protein transgenic founder animals are produced (i.e. those animals which express target proteins in cells or tissues of interest and which, preferably, exhibit symptoms of pancreatic islet and/or β -cell disorders), they may be bred, inbred, outbred or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to, outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound target protein transgenics that transgenically express the target protein of interest at higher levels because of the effects of additive expression of each target gene transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment

expression and eliminate the possible need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the target protein and the development of pancreatic islet and/or β -cell disorder-like symptoms. One such approach is to cross the target protein transgenic founder animals with a wild type strain to produce an F1 generation that exhibits pancreatic islet and/or β -cell disorder-like symptoms, such as glucose intolerance, hyperinsulinaemia, non-insulin dependent diabetes and obesity. The F1 generation may then be inbred in order to develop a homozygous line, if it is found that homozygous target protein transgenic animals are viable.

2.2.2 Cell-based assays

Cells that contain and express target gene sequences which encode target proteins and, further, exhibit cellular phenotypes associated with an insulin resistance disorder, may be utilised to identify compounds that exhibit an ability to ameliorate pancreatic islet and/or β -cell disorder symptoms. Cellular phenotypes, which may indicate an ability to ameliorate pancreatic islet and/or β -cell disorders, may include, for example, abnormal insulin release in response to a glucose or glyceraldehyde stimulus.

Further, the fingerprint pattern of protein expression of cells of interest may be analysed and compared to the normal fingerprint pattern. Those compounds which cause cells exhibiting pancreatic islets and/or β -cell

disorder-like cellular phenotypes to produce a fingerprint pattern more closely resembling a normal fingerprint pattern for the cell of interest may be considered candidates for further testing regarding an ability to ameliorate pancreatic islet and/or β -cell disorder symptoms.

Cells which can be utilised for such assays may, for example, include non-recombinant cell lines, such as Rin m5F, Rin 5F, Hit 15 and BRINS insulinoma cell lines.

Further, cell lines which may be used for such assays may also include recombinant, transgenic cell lines. For example, the pancreatic islet and/or β -cell disorder animal models of the invention discussed above, in Section 2.2.1, may be used to generate cell lines, containing one or more cell types involved in pancreatic islet cell disorders, that can be used as cell culture models for this disorder. While primary cultures derived from the pancreatic islet cell disorder transgenic animals of the invention may be utilised, the generation of continuous cell lines is preferred. For examples of techniques which may be used to derive a continuous cell line from the transgenic animals; see Small, et al., 1985, Mol. Cell Biol. 5: 642-648.

Alternatively, cells of a cell type known to be involved in pancreatic islet cell disorders may be transfected with sequences capable of increasing or decreasing the amount of target protein within the cell. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the cell of interest, or, if endogenous gene sequences of the target protein

are present, they may either be overexpressed or, alternatively, be disrupted in order to underexpress or inactivate target protein expression.

5 In order to overexpress a gene sequence of a target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the cell type of interest. Such regulatory regions will be well known to those of
10 skill in the art, and may be utilised in the absence of undue experimentation.

For underexpression of an endogenous target protein the gene sequence may be isolated and engineered such that
15 when reintroduced into the genome of the cell type of interest, the endogenous target gene alleles will be inactivated. Preferably, the engineered target gene sequence is introduced via gene targeting such that the endogenous target sequence is disrupted upon integration
20 of the engineered target gene sequence into the cell's genome. Gene targeting is discussed above, in Section 2.2.1.

Transfection of target protein gene sequence nucleic acid
25 may be accomplished by utilising standard techniques. See, for example, Ausubel, 1989, supra. Transfected cells should be evaluated for the presence of the recombinant target gene sequences, for expression and accumulation of target gene mRNA, and for the presence of
30 recombinant target protein production. In instances wherein a decrease in target protein expression is desired, standard techniques may be used to demonstrate whether a decrease in endogenous target gene expression

and/or in target protein production is achieved.

3 Screening assays for compounds that interact with
 the target proteins

5 The following assays are designed to identify compounds
 that bind to target proteins, bind to other cellular
 proteins that interact with target proteins, and to
 compounds that interfere with the interaction of the
10 target proteins with other cellular proteins. Such
 compounds may include, but are not limited to, other
 cellular proteins. Methods for the identification of
 such cellular proteins are described below in Section 3.2

15 Compounds may include, but are not limited to, peptides
 such as, for example, soluble peptides, including, but
 not limited to, Ig-tailed fusion peptides, comprising
 extracellular portions of target protein transmembrane
 receptors, and members of random peptide libraries. (see,
20 e.g. Lam, K.S. et al., 1991, Nature 354: 82-84;
 Houghten, R. et al., 1991, Nature 354: 84-86) made of D-
 and/or L-configuration amino acids, phosphopeptides
 (including, but not limited to, member of random or
 partially degenerate, directed phosphopeptide libraries:
 see, e.g., Songyang, Z. et al., 1993, Cell 72: 767-778),
25 antibodies (including, but not limited to, polyclonal,
 monoclonal, humanised, anti-idiotypic, chimeric or single
 chain antibodies, and FAb, F(ab')₂ and FAb expression
 library fragments, and epitope-binding fragments thereof)
 and small organic or inorganic molecules.

30 Compounds identified via assays such as those described
 herein may be useful, for example, in elaborating the
 biological function of the target protein, and for

ameliorating pancreatic islet and/or β -cell disorders. In instances, for example, whereby a pancreatic islet and/or β -cell disorder situation results from a lower overall level of target protein expression and/or target protein activity in a cell or tissue involved in such a pancreatic islet and/or β -cell disorder, compounds that interact with the target protein may include ones which accentuate or amplify the activity of the bound target protein. Such compounds would bring about an effective increase in the level of target protein activity, thus ameliorating symptoms. In instances whereby mutations within the target gene cause aberrant target proteins to be made which have a deleterious effect that leads to a pancreatic islet and/or β -cell disorder, compounds that bind target protein may be identified that inhibit the activity of the bound target protein. Assays for testing the effectiveness of compounds, identified by, for example, techniques such as those described in Sections 3.1 to 3.3, are discussed below, in Section 3.4.

3.1 In vitro screening assays for compounds that bind to the target proteins

In vitro systems may be designed to identify compounds capable of binding the target proteins of the invention. Compounds identified may be useful, for example, in modulating the activity of wild type and/or mutant target proteins, may be useful in elaborating the biological function of the target protein, may be utilised in screens for identifying compounds that disrupt normal target protein interactions, or may in themselves disrupt such interactions.

The principle of the assays used to identify compounds

that bind to the target protein involves preparing a reaction mixture of the target protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring target protein or the test substance onto a solid phase and detecting target protein/test compounds complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the target protein may be anchored onto a solid surface, and the test compound, which is not anchored, may be labelled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilised as the solid phase. The anchored component may be immobilised by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilised antibody, preferably a monoclonal antibody, specific for the protein to be immobilised may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the non-immobilised component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g. by washing) under conditions such that any complexes formed will remain immobilised on the solid surface. The detection of complexes anchored on the solid surface can be

accomplished in a number of ways. Where the previously non-immobilised component is pre-labelled, the detection of label immobilised on the surface indicates that complexes were formed. Where the previously non-immobilised component is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface, e.g. using a labelled antibody specific for the previously non-immobilised component (the antibody, in turn, may be directly labelled or indirectly labelled with a labelled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected, e.g. using an immobilised antibody specific for target protein or the test compound to anchor any complexes formed in solution, and a labelled antibody specific for the other component of the possible complex to detect anchored complexes.

3.2 Assays for cellular proteins that interact with the target protein

Any method suitable for detecting protein-protein interactions may be employed for identifying novel target protein-cellular or extracellular protein interactions.

These methods are outlined in Section 1.2 for the identification of pathway proteins, and may be utilised herein with respect to the identification of proteins which interact with identified target proteins.

3.3 Assays for compounds that interfere with target protein/cellular macromolecule interaction

The target proteins of the invention may, in vivo, interact with one or more cellular or extracellular

macromolecules, such as proteins. Such macromolecules may include, but are not limited to, nucleic acid molecules and those proteins identified via methods such as those described above in Section 3.2. For purposes of this discussion, such cellular and extracellular macromolecules are referred to herein as 'binding partners'. Compounds that disrupt such interactions may be useful in regulating the activity of the target protein, especially mutant target proteins. Such compounds may include, but are not limited to, molecules such as antibodies, peptides, and the like, as described, for example, in Section 3.1.

The basic principle of the assay systems used to identify compounds that interfere with the interaction between the target protein and its cellular or extracellular binding partner or partners involves preparing a reaction mixture containing the target protein, and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of target protein and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the target protein and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the

interaction of the target protein and the interactive binding partner. Additionally, complex formation within reaction mixtures contains the test compound and a mutant target protein. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal target proteins.

The assay for compounds that interfere with the interaction of the target and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the target protein or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the target protein and the binding partners, e.g. by competition, can be identified by conducting the reaction in the presence of the test substance, i.e. by adding the test substance to the reaction mixture prior to or simultaneously with the target protein and interactive cellular or extracellular binding partner. Alternatively, test compounds that disrupt pre-formed complexes, e.g. compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the target protein or the interactive cellular or extracellular binding partner, is anchored onto a solid surface, while the non-anchored species is labelled, either directly or indirectly. In practice, microtiter plates are conveniently utilised. The anchored species may be immobilised by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the target gene product or binding partner and drying.

Alternatively, an immobilised antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilised species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g. by washing) and any complexes formed will remain immobilised on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilised species is pre-labelled, the detection of label immobilised on the surface indicates that complexes were formed. Where the non-immobilised species is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface, e.g. using a labelled antibody specific for the initially non-immobilised species (the antibody, in turn, may be directly labelled or indirectly labelled with a labelled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt pre-formed

complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected, e.g. using an immobilised antibody specific for one of the binding components to anchor any complexes formed in solution, and a labelled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt pre-formed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a pre-formed complex of the target protein and the interactive cellular or extracellular binding partner is prepared in which either the target protein or its binding partners is labelled, but the signal generated by the label is quenched due to complex formation (see, e.g. US Patent No. 4,109,496 by Rubenstein which utilises this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the pre-formed complex will result in the generation of a signal above background. In this way, test substances, which disrupt target protein/cellular or extracellular binding partner interaction, can be identified.

In a particular embodiment, the target protein can be prepared for immobilisation using recombinant DNA techniques described in Section 2.1. For example, the target protein gene coding region can be fused to a

glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive cellular or extracellular binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practised in the art and described above, in Section 2.1. This antibody can be labelled with the radioactive isotope ^{125}I , for example, by methods routinely practised in the art. In a heterogeneous assay, e.g. the GST-target protein gene fusion protein can be anchored to glutathione-agarose beads. The interactive cellular or extracellular binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labelled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the target protein and the interactive cellular or extracellular binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST-target protein gene fusion protein and the interactive cellular or extracellular binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is

washed away. Again the extent of inhibition of the target protein/binding partner interaction can be detected by adding the labelled antibody and measuring the radioactivity associated with the beads.

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In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the target protein and/or the interactive cellular or extracellular binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely practised in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in this Section above, and allowed to interact with and bind to its labelled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labelled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the cellular or extracellular binding partner is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding

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activity and purified or synthesised.

For example, and not by way of limitation, a target protein can be anchored to a solid material as described above, in this Section by making a GST-target protein gene fusion protein and allowing it to bind to glutathione agarose beads. The interactive cellular or extracellular binding partner can be labelled with a radioactive isotope, such as ^{35}S , and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-target protein gene fusion protein and allowed to bind. After washing away unbound peptides, labelled bound material, representing the cellular or extracellular binding partner binding domain, can be eluted, purified and analysed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

3.4 Assays for amelioration of pancreatic islet and/or β -cell disorder symptoms

Any of the binding compounds, including but not limited to, compounds such as those identified in the foregoing assay systems, may be tested for the ability to prevent or ameliorate pancreatic islet and/or β -cell disorder symptoms, which may include, for example, non-insulin dependent diabetes. Cell-based and animal model-based assays for the identification of compounds exhibiting such an ability to ameliorate pancreatic islet and/or β -cell disorder symptoms are described below.

First, cell-based systems such as those described above,

in Section 2.2.2, may be used to identify compounds, which may act to prevent or ameliorate pancreatic islet and/or β -cell disorder symptoms. For example, such cell systems may be exposed to a compound, suspected of exhibiting an ability to ameliorate pancreatic islet and/or β -cell disorder symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of pancreatic islet and/or β -cell disorder symptoms in the exposed cells. After exposure, the cells are examined to determine whether one or more of the pancreatic islet and/or β -cell disorder-like cellular phenotypes has been altered to resemble a more normal or more wild type phenotype, or a phenotype more likely to produce a lower incidence or severity of disorder symptoms.

In addition, animals-based pancreatic islet and/or β -cell disorder systems, such as those described above, In Section 2.2.1, may be used to identify compounds capable of ameliorating pancreatic islet and/or β -cell disorder-like symptoms. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions which may be effective in treating such disorders. For example, animal models may be exposed to a compound, suspected of exhibiting an ability to prevent or ameliorate pancreatic islet and/or β -cell disorder symptoms, at a sufficient concentration and for a time sufficient to elicit such a prevention or amelioration of the pancreatic islet and/or β -cell disorder symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with pancreatic islet and/or β -cell disorders such as non-

insulin dependent diabetes.

With regard to intervention, any treatments that reverse any aspect of pancreatic islet and/or β -cell disorder-like symptoms should be considered as candidates for human pancreatic islet and/or β -cell disorder therapeutic intervention including the treatment of non-insulin dependent diabetes. Dosages of test agents may be determined by deriving dose-response curves, as discussed in Section 6.1 below.

Similarly, any treatments that can prevent the development of pancreatic islet and/or β -cell disorders should be considered as candidates for the prevention of human pancreatic islet and/or β -cell disorder therapeutic intervention. Such disorders include, but are not limited to, NIDDM.

Protein expression patterns may be utilised in conjunction with either cell-based or animal-based systems to assess the ability of a compound to ameliorate pancreatic islet and/or β -cell disorder-like symptoms. For example, the expression pattern of one or more fingerprint proteins may form part of a fingerprint profile, which may then be used in such as assessment. Fingerprint profiles are described below, in Section 7.1. Fingerprint profiles may be characterised for known states, either pancreatic islet and/or β -cell disorder or normal states, within the cell- and/or animal-based model systems. Subsequently, these known fingerprint profiles may be compared to ascertain the effect a test compound has to modify such fingerprint profiles, and to cause the profile to more closely resemble that of a more desirable

fingerprint. For example, administration of a compound may cause the fingerprint profile of a pancreatic islet and/or β -cell disorder model system to more closely resemble the control system. Administration of a compound may, alternatively, cause the fingerprint profile of a control system to begin to mimic an pancreatic islet and/or β -cell disorder state, which may, for example, be used in further characterising the compound of interest, or may be used in the generation of additional animal models.

4. Compounds and methods for treatment of pancreatic islet and/or β -cell disorders

Described below are methods and compositions whereby pancreatic islet and/or β -cell disorder symptoms may be ameliorated. It is possible that pancreatic islet and/or β -cell disorders may be brought about, at least in part, by an abnormal level of target protein, or by the presence of a target protein exhibiting an abnormal activity. As such, the reduction in the level and/or activity of such target protein would bring about the amelioration of pancreatic islet and/or β -cell disorder-like symptoms. Techniques for the reduction of target protein gene expression levels or target protein activity levels are discussed in Section 4.1.

Alternatively, it is possible that pancreatic islet and/or β -cell disorders may be brought about, at least in part, by the absence or reduction of the level of target protein expression, or a reduction in the level of a target protein's activity. As such, an increase in the level of target protein gene expression and/or the activity of such proteins would bring about the

amelioration of pancreatic islet and/or β -cell disorder-like symptoms. Techniques for increasing target protein gene expression levels or target protein activity levels are discussed in Section 4.2.

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4.1 Compounds that inhibit expression, synthesis or activity of mutant target proteins

As discussed above, target proteins involved in pancreatic islet and/or β -cell disorders may cause such disorders via an increased level of target protein activity. A variety of techniques may be utilised to inhibit the expression, synthesis, or activity of such target genes and/or proteins.

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For example, compounds such as those identified through assays described above, in Section 3, which exhibit inhibitory activity, may be used in accordance with the invention to prevent or ameliorate pancreatic islet and/or β -cell disorder symptoms. As discussed in Section 3 above, such molecules may include, but are not limited to, peptides (such as, for example, peptides representing soluble extracellular portions of target protein transmembrane receptors), phosphopeptides, small organic or inorganic molecules, or antibodies (including, for

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example, polyclonal, monoclonal, humanised, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab'), and FAb expression library fragments, and epitope-binding fragments thereof). Techniques for determination of effective doses and administration of such compounds are described below, in Section 6.1. Inhibitory antibody techniques are further described below, in Section 4.1.2.

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Further, antisense and ribosome molecules, which inhibit

expression of the target protein gene, may also be used in accordance with the invention to inhibit the aberrant target protein gene activity. Such techniques are described below, in Section 4.1.1; triple helix molecules may be utilised in inhibiting the aberrant target protein gene activity.

4.1.1 Inhibitory antisense, ribosome and triple helix approaches

Among the compounds, which may exhibit the ability to prevent or ameliorate pancreatic islet and/or β -cell disorder symptoms are antisense, ribosome and triple helix molecules. Such molecules may be designed to reduce or inhibit either wild type, or if appropriate, mutant target protein gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridising to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxy-ribonucleotides derived from the translation initiation site, e.g. between the -10 and +10 regions of the target gene nucleotide sequence of interest, are preferred.

Ribosomes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. (For a review, see Rossi, J., 1994, Current Biology 4: 469-471). The mechanism of ribosome action involves sequence specific hybridisation of the ribosome molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribosome molecules must include one or

more sequences complementary to the target protein mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see US Patent No:5,093,246. As such, within the scope of the invention are engineered hammerhead motif ribosome molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding target proteins.

Specific ribosome cleavage sites within any potential RNA target are initially identified by scanning the molecule of interest for ribosome cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short TNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target protein gene, containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridise with complementary oligonucleotides, using ribonuclease protection assays.

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the

resulting triple helix. The pyrimidine-rich molecules provide base complementary to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be
5 chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in
10 GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule.
15 Switchback molecules are synthesised in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

20 Anti-sense RNA and DNA, ribosome and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. They include techniques for chemically
25 synthesising oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as, for example, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the
30 antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors, which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense

cDNA constructs that synthesise antisense RNA constitutively inducibly, depending on the promoter used, can be introduced stably into cell lines.

5 Various well-known modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences or ribo- or deoxy-nucleotides to the 5' and/ or 3' ends
10 of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxy-ribonucleotide backbone.

4.1.2 Antibodies for the inhibition of target protein

15 Antibodies that are both specific for target protein and interfere with its activity may be used to inhibit target protein function. Where desirable, antibodies specific for mutant target protein, which interferes with the activity of such mutant target product, may also be used.
20 Such antibodies may be generated using standard techniques described in Section 2.3, supra, against the proteins themselves or against peptides corresponding to portions of the proteins. The antibodies include, but are not limited to, polyclonal, monoclonal, Fab
25 fragments, single chain antibodies, chimeric antibodies, etc.

In instances where the target gene protein is intracellular and whole antibodies are used,
30 internalising antibodies may be preferred. However, lipofectin or liposomes may be used to deliver the antibody or a fragment of the Fab region, which binds to the target protein epitope into cells. Where fragments

of the antibody are used, the smallest inhibitory fragment, which binds to the target protein's binding domain, is preferred. For example, peptides having an amino acid sequence corresponding to the domain of the variable region of the antibody that binds to the target protein may be used. Such peptides may be synthesised chemically or produced via recombinant DNA technology using methods well known in the art (e.g. see Creighton, 1983, supra; and Sambrook et al, 1989, supra).

Alternatively, single chain neutralising antibodies, which bind to intracellular target protein epitopes, may also be administered. Such single chain antibodies may be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell populating by utilising, for example, techniques such as those described in Marasco et al (Marasco, W. et al, 1993, Proc. Natl. Acad. Sci. USA, 90: 7889-7893).

In instances where the target protein is extracellular, or is a transmembrane protein, any of the administration techniques described below, in Section 6, which are appropriate for peptide administration may be utilised to effectively administer inhibitory target protein antibodies to their site of action.

4.2 Methods for restoring target protein activity

Target proteins that cause pancreatic islet and/or β -cell disorders may be underexpressed within pancreatic islet and/or β -cell disorder situations. Alternatively, the activity of target protein may be diminished, leading to the development of pancreatic islet and/or β -cell

disorder symptoms. Described in this Section are methods whereby the level of target protein may be increased to levels wherein pancreatic islet and/or β -cell disorder symptoms are prevented or ameliorated. The level of target protein activity may be increased, for example, by either increasing the level of target protein present or by increasing the level of active target protein, which is present.

For example, a target protein, at a level sufficient to ameliorate pancreatic islet and/or β -cell disorder symptoms may be administered to a patient exhibiting such symptoms. Any of the techniques discussed below, in Section 6, may be utilised for such administration. One of skill in the art will readily know how to determine the concentration of effective, non-toxic doses of the normal target protein, utilising techniques such as those described below, in Section 4.6.1.

Further, patients may be treated by gene replacement therapy. One or more copies of a normal target protein gene or a portion of the gene that directs the production of a normal target protein with target protein gene function, may be inserted into cells, using vectors which include, but are not limited to, adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes. Additionally, techniques such as those described above may be utilised for the introduction of normal target protein gene sequences into human cells.

Cells, preferably autologous cells, containing normal target protein gene sequences may then be introduced or

reintroduced into the patient at positions which allow for the prevention or amelioration of pancreatic islet and/or β -cell disorder symptoms. Such cell replacement techniques may be preferred, for example, when the target protein is a secreted, extracellular protein.

Additionally, antibodies may be administered which specifically bind to a target protein and by binding, serve to, either directly or indirectly, activate the target protein function. Such antibodies can include, but are not limited to, polyclonal, monoclonal, FAb fragments, single chain antibodies, chimeric antibodies and the like. The antibodies may be generated using standard techniques such as those described above, in Section 2.3, and may be generated against the protein themselves or against proteins corresponding to portions of the proteins. The antibodies may be administered, for example, according to the techniques described above, in Section 4.1.2.

5. Pharmaceutical preparations and methods of administration

The identified compounds, nucleic acid molecules and cells that affect target protein expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to prevent or to treat or to ameliorate pancreatic islet and/or β -cell disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of pancreatic islet and/or β -cell disorder, including non-insulin dependent diabetes, or alternatively, to that amount of a nucleic acid molecule

sufficient to express a concentration of protein which results in the amelioration of such symptoms.

5.1 Effective dose

5 Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining by ED_{50} (the dose therapeutically effective in 50% of the population) and by determining the ED_{50} of any
10 side-effects (toxicity - TD_{50}). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio TD_{50}/ED_{50} . Compounds, which exhibit large therapeutic indices, are preferred. While compounds that exhibit toxic side effects may be
15 used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimise potential damage to uninfected cells and, thereby, reduce side effects.

20 The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The
25 dosage may vary within this range depending upon the dosage form employed and the route of administration utilised.

5.2 Formulations and use

30 Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral and rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pre-gelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methyl-cellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium, stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active

compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurised aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g. gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g. by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g. in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other glycerides.

5

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation, for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

10

15

The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as blister pack. The pack or dispenser device may be accompanied by instructions for administration.

20

7. Diagnosis of pancreatic islet and/or β -cell disorder abnormalities including non-insulin dependent diabetes

25

A variety of methods may be employed for the diagnosis of pancreatic islet and/or β -cell disorders, the predisposition to pancreatic islet and/or β -cell disorders, and for monitoring the efficacy of any pancreatic islet and/or β -cell disorder compounds during, for example, clinical trials and for monitoring patients undergoing clinical evaluation for the treatment of such

30

insulin resistance disorders.

The methods include conventional methods such as measurements of glucose/insulin ratios in whole animals or during glucose tolerance tests, e-peptide in urine or plasma.

Non-insulin dependent diabetes may be detected and the efficacy of treatment monitored by methods and parameters identified by such bodies as the World Health Organisation, the International Diabetes Federation and the American Diabetes Association.

Methods may also, for example, utilise reagents such as the fingerprint protein described in Section 1, and antibodies directed against differentially expressed and pathway proteins, as described above, in Sections 1.3 (peptides) and 2.3 (antibodies). Specifically, such reagents may be used for (1) the detection of the presence of target protein mutations, or (2) the detection of either an over- or an under-abundance of target protein relative to the normal state.

The methods described herein may be performed, for example, by utilising pre-packaged diagnostic kits comprising at least one specific finger print protein or anti-fingerprint protein antibody reagent described herein, which may be conveniently used, e.g. in clinical settings, to diagnose patients exhibiting pancreatic islet and/or β -cell disorder abnormalities.

Any cell type or tissue in which the fingerprint protein is expressed may be utilised in the diagnostics described

below. Examples of suitable samples types include cell samples, tissue samples, and fluid samples such as blood urine or plasma.

5 Among the methods, which can be utilised herein, are methods for monitoring the efficacy of compounds in clinical trials for the treatment of pancreatic islet and/or β -cell disorders. Such compounds can, for example, be compounds such as those described above, in
10 Section 4. Such a method comprises detecting, in a patient sample, a protein, which is differentially expressed in the pancreatic islet and/or β -cell disorder state relative to its expression in a normal state.

15 During clinical trials, for example, the expression of a single fingerprint protein, or alternatively, a fingerprint pattern of a cell involved in a pancreatic islet and/or β -cell disorder can be determined in the presence or absence of the compound being tested. The
20 efficacy of the compound can be followed by comparing the expression data obtained to the corresponding known expression patterns in a normal state. Compounds exhibiting efficacy are those which alter the single fingerprint protein expression and/or the fingerprint
25 pattern to more closely resemble that of the normal state.

The detection of the protein differentially expressed in an pancreatic islet and/or β -cell disorder state relative
30 to their expression in a normal state can also be used for monitoring the efficacy of potential pancreatic islet and/or β -cell disorder compounds and compounds for the treatment of non-insulin dependent diabetes during

clinical trials. During clinical trials, for example, the level and/or activity of the differentially expressed protein can be determined in relevant cells and/or tissues in the presence or absence of the compound being tested. The efficacy of the compound can be followed by comparing the protein level and/or activity data obtained to the corresponding known levels/activities for the cells and/or tissues in a normal state. Compounds exhibiting efficacy are those which alter the pattern of the cell and/or tissue involved in the pancreatic islet and/or β -cell disorder to more closely resemble that of the normal state.

Example 1:

Mouse treatment protocol

Lean and obese female C57 B1/6J ob/ob mice were given BRL 49653, rosiglitazone, 10mg/kg/day, by oral gavage for 7 days. This treatment produced significant improvement in oral glucose tolerance and insulin sensitivity in ob/ob mice, but had no effect in the lean litter mates. Non-fasting animals were anaesthetised with 50% "Hyponovel" and 50% "Hyponorm" and then killed humanely with carbon dioxide gas. The pancreas was then removed.

Preparation of pancreatic islets

Pancreatic islets were isolated by collagenase digestion (collagenase type VI, Sigma, UK) at 37°C using a physiological saline solution supplemented with 1mM CaCl_2 , 4mM glucose and equilibrated with $\text{CO}_2:\text{O}_2$ (5%:95%), pH 7.4. Islets were hand-picked using a binocular microscope. The islets were washed and further microdissected in order to remove residual acinar material.

The islets were then snap frozen in liquid N₂ and stored frozen.

Protein solubilisation

5 For analytical 2-D-PAGE, 125 pancreatic islets were mixed with 60 microlitres of a solution containing urea (8M), CHAPS (4% w/v), Tris (40mM), DTE (65mM), SDS (0.05% w/v) and a trace of bromophenol blue. A weighed portion of the final diluted sample was loaded into a sample cup at
10 the cathodic end of the IPG gels.

First dimension electrophoresis

A non-linear immobilised pH gradient of IPG strips (3.5-10.0 NL IPG 18cm) was used as the first dimension. It
15 offered high resolution, great reproducibility and allowed high protein loads. Based on specifications of the Geneva University Hospital, the non-linear pH gradient strips were prepared by Amersham-Pharmacia Biotechnology AB and are commercially available. The
20 strips were 3mm wide and 180mm long.

Hydration of the IPG strips was performed overnight in a Pharmacia reswelling cassette with 25ml of a solution of urea (8M), CHAPS (2% w/v), DTE (10mM), Resolyte pH 3.5-10
25 (2% v/v) and a trace of bromophenol blue.

When the rehydration cassette had been thoroughly emptied and opened, the strips were transferred to the Pharmacia strip tray. After placing IPG strips, humid electrode
30 wicks, electrodes and sample cups in position, the strips and cups were covered with low viscosity paraffin oil. Samples were applied in the cups at the cathodic end of the IPG strips in a slow and continuous manner, without

touching the gel.

The voltage was linearly increased from 300 to 3500 V during 3 hours, followed by 3 additional hours at 3500 V, whereupon the voltage was increased to 5000 V. A total volt hour product of 100kvh was used in an overnight run.

Second dimension of the electrophoresis

After the first dimension run, the IPG strips were equilibrated in order to resolubilise the proteins and to reduce -S-S- bonds. The strips were thus equilibrated within the strip tray with 100ml of a solution containing Tris-HCl (50mM), pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 min. The SH groups were subsequently blocked with 100 ml of a solution containing Tris-HCl (50mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of bromophenol blue for 5 min.

In the second dimension run, a vertical gradient slab gel with the Laemmli-SDS-discontinuous system was used with some small modifications, which may be summarised as follows:

- Gels are not polymerised in the presence of SDS. This seems to prevent the formation of micelles, which contain acrylamide monomer, thus increasing the homogeneity of pore size and reducing the concentration of unpolymerised monomer in the polyacrylamide. The SDS used in the gel running buffer is sufficient to maintain the necessary negative charge on proteins.

- Piperazine-diacrylyl (PDA) is used as crosslinker. This is believed to reduce N-terminal protein blockage, gives better protein resolution, and reduces diammine silver staining background.
- Sodium thiosulphate is used as an additive to reduce background in the silver staining of gels.
- The combination of the IPG strip and agarose avoids the need for a stacking gel.

The gel composition and dimensions were as follows:

Dimensions:	160 x 200 x 1.5mm
Resolving gel:	Acrylamide/PDA (9-16% T/2.6% C)
Stacking gel:	No stacking
Leading buffer:	Tris-HCl (0.375M) pH 8.8
Trailing buffer:	Tris-glycine-SDS (25mM-198mM-0.1% w/v) pH 8.3
Additives:	Sodium thiosulphate (5mM)
Polymerisation agents:	TEMED (0.05%) APS (0.1%)

The gels were poured until 0.7cm from the top of the plates and over-layered with sec-butanol for about two hours. After the removal of the overlay and its replacement with water the gels were left overnight.

After the equilibration, the IPG gel strips were cut to size. Six mm were removed from the anodic end and 14mm from the cathodic end. The second dimension gels were over-layered with a solution containing agarose (0.5% w/v) and Tris-glycine-SDS (25mM-198mM-0.1% w/v) pH 8.3 heated at about 70°C and the IPG gel strips were immediately loaded through it.

The gel was run at 8-12°C for 5 hours at a constant current of 40mA/gel. The voltage is non-limiting, but usually requires 100 to 400 V.

5 Staining

Silver staining, which is 100-fold more sensitive than Coomassie Brilliant Blue staining, was used (except where otherwise stated). Thus, the 2-DGE gels were stained with an ammoniacal silver staining as follows:

10

All steps were performed on an orbital shaker at 36 rpm.

Step 1: At the end of the second dimension run, the gels were removed from the glass plates and washed in deionised water for 5 min.

15

Step 2: The gels were soaked in ethanol:acetic acid:water (40:10:50 volume ratio) for 1 hour.

Step 3: The gels were soaked in ethanol:acetic acid:water (5:5:90 volume ratio) for 2 hours or overnight.

20

Step 4: They were washed in deionised water for 5 min.

Step 5: They were soaked in a solution containing glutaraldehyde (1% w/v and sodium acetate (0.5M) for 30 min.

Step 6: They were washed 3 times in deionised water for

25

10 min.

Step 7: In order to obtain homogenous dark brown staining of proteins, gels were soaked twice in a 2, 7-naphthalenedisulphonic acid solution (0.05% w/v) for 30 min.

30

Step 8: The gels were then rinsed 4 times in deionised water for 15 min.

Step 9: The gels were stained in a freshly made ammoniacal silver nitrate solution for 30 minutes. To

prepare 750ml of this solution, 6g of silver nitrate were dissolved in 30ml of deionised water, which was slowly mixed into a solution containing 160ml of water, 10ml of concentrated ammonia (25%) and 1.5ml of sodium hydroxide (10N). A transient brown precipitate might form. After it cleared, water was added to give the final volume.

Step 10: After staining, the gels were washed 4 times in deionised water for 4 min.

Step 11: The images were developed in a solution of citric acid (0.01% w/v) and formaldehyde (0.1% v/v) for 5 to 10 min.

Step 12: When a slight background stain appeared, development was stopped with a solution of Tris (5% w/v) and acetic acid (2% v/v).

Scanning of the gels

The Laser Densitometer (4000 x 5000 pixels; 12 bits/pixel) from Molecular Dynamics and the GS-700 from Bio-Rad have been used as scanning devices. These scanners were linked to "Sparc" workstations and "Macintosh" computers.

Quantitative image analysis of the gels using "Melanie II"

Two-dimensional polyacrylamide gels may be digitised and analysed by computer to allow quantitative image analysis and automatic gel comparison. Since the 2-D-PAGE technique was first developed in 1975 several computer systems have been manufactured, mainly by academic 2-D-PAGE related laboratories. In the present work, "Melanie II", developed at the University Hospital of Geneva was used. It is available for "Unix" workstations, as well as for "Power Macintosh" and IBM-compatible computers.

ob/ob and lean mice spot detection, quantitation and matching, gel image extraction, zooming, warping and printing as well as gel stacking and flipping were carried out with the "MelView" program.

5

The images were then classified in four classes; lean control, ob/ob control, lean treated and ob/ob treated. Differential analysis and the Student T test, using the relative abundance of each spot (% volume), allowed the
10 detection of significant ($p < 0.01$) over- and under-expressed polypeptides.

Results

The following DEPs were found:

15

Group 1. POM6, POM7, POM8, POM9 and POM10 all had a reduced expression in islets from ob/ob control mice relative to their expression in islets from lean control mice. Their expression in ob/ob islets was not increased by treatment with rosiglitazone.

20

Group 2. POM(T)1, POM(T)2, POM(T)4 all had a reduced expression in islets from ob/ob control mice relative to the expression in lean control mice and their expression in ob/ob mice were increased towards the level in lean
25 control mice following treatment with rosiglitazone.

30

POM(T)3, POM(T)5, POM(T)11, POM(T)12 and POM(T)13 all had an increased expression in islets from ob/ob control mice relative to the level in lean control mice and treatment with rosiglitazone decreased their expression towards that in lean control mice. Thus treatment of ob/ob mice with rosiglitazone, which improves diabetic control in these mice, restored the expression of these proteins in

ob/ob mice towards the expression level in lean mice. In each case, similar treatment of lean mice with rosiglitazone had no effect on the expression level and therefore the alteration in pancreatic islet protein expression appears to be related to improved pancreatic islet function associated with the rosiglitazone treatment.

Group 3. Two proteins PSEM 14 and PSEM 15 did not show any differential expression between lean control and ob/ob control mice but treatment with rosiglitazone increased the expression of these proteins in both lean and ob/ob mice. Thus these DEPs may represent an activity of rosiglitazone unrelated to its effect in improving pancreatic islet cell function.

The drawings are presented to show the location of the DEPs. Fig. 1 shows the 2D maps of proteins from the pancreatic islets of lean control mice. Figs. 2-7 and Figs. 9-13 show the comparative expression of the DEPs together with quantification of the differential expression. With comparative purposes Figs. 8 and 14 show a control protein (C1230) that is not differentially expressed.

Preparative 2-D-PAGE

The analytical 2-DGE described above was repeated, using larger numbers of islets.

After the first dimension run the strips were equilibrated using 3ml of each buffer per groove.

Protein electroblotting

The blotting of proteins separated by 2-D-PAGE onto

polyvinylidene difluoride (PVDF) membranes has enabled the identification and characterisation of proteins from complex biological samples. Transfer of the proteins can be carried out using several methods such as vacuum, capillary or electric field. Electroblotting, using vertical buffer tanks or a semi-dry method, is preferred. Both techniques can use the 3-[cyclohexamino]-1-propanesulfonic acid (CAPS) transfer buffer. Gloves must be worn and all filter papers should be washed three times for 3 min in water three times in transfer buffer. These two steps are important in order to avoid any protein or amino acid contamination.

The procedure was as follows. After second dimensional electrophoresis, the gels were soaked in deionised water for 3 min. Then they were equilibrated in a solution containing 10mM CAPS pH 11 for 30 min. At the same time, PVDF membranes were wetted in methanol for 1 min and equilibrated in a solution containing 10mM CAPS pH 11 and methanol (10% v/v) also for 30 min. Electroblotting was carried out in a semi-dry apparatus with a solution containing 10mM CAPS pH 11 and methanol (20% v/v anodic side; 5% v/v cathodic side) at 1 mA/cm² constant current for 3 hours at 15°C.

Protein detection on PVDF membranes

Amido Black and Coomassie Brilliant Blue R-250 were used instead of silver staining to visualise proteins on PVDF membranes and are compatible with the ensuing post-separation analysis. Thus, in another 2-DGE run, after electrotransfer, the PVDF membranes were stained in a solution containing Amido Black (0.5% w/v, isopropanol (25% v/v) and acetic acid (10% v/v) for 2 min.

Destaining was done by several soakings in deionised water.

5 In another run, after electrotransfer, the PVDF membranes were stained in a solution containing Coomassie Brilliant Blue R-250 (0.1% w/v) and methanol (50% v/v) for 15 min. Destaining was done in a solution containing methanol (40% v/v) and acetic acid (10% v/v). The same method was used for preparative gels that did not need
10 electrotransfer for further post-separation analysis, such as peptide mass fingerprinting.

The PVDF stained membranes were either air-dried or dried on a 3mm thick plate onto a heating plate at 37°C for 10
15 min.

Scanning

This was done as described above.

Protein identification

20 In amino sequence analysis by Edman degradation, amino acid derivatives are sequentially cleaved one at a time from the protein. Proteins with a chemically inaccessible alpha-amino group cannot be sequenced
25 directly by this procedure and are termed N-terminally blocked. The best way to overcome the blocked proteins is to generate individual fragments by chemical or proteolytic cleavage. Routinely, ten to twelve Edman degradation cycles were performed for each spot. A
30 search in the SWISS-PROT database was made to detect identity to known protein sequences.

The Amido Black stained proteins were excised with a

razor blade and N-terminal sequencing was performed using an ABI model 473A or 477A microsequencer from Applied Biosystems equipped with "Problott" cartridges.

5 For internal sequencing, the spots of interest were excised and soaked for two hours in a solution containing acetic acid (100mM), methanol (10% v/v) and PVP-40 (1% v/v) at 37°C. After three washes in deionised water, the PVDF spots were cut into small pieces (about 1 mm²) and
10 incubated in 25 microlitres of a solution containing sodium phosphate (100mM) pH 8.0 and lysyl endopeptidase (1 microgram). Following overnight digestion a room temperature, guanidine-HCl (28mg) and DTT (100 micrograms) were added. After reduction for 2 hours at
15 37°C, the mixture was incubated for 30 min, at room temperature, with 300 micrograms of iodoacetamide. The digestion solution was removed and kept. PVDF pieces were then extracted overnight with 25 microlitres of a solution containing isopropanol (70% v/v) and
20 trifluoroacetic acid (5% w/v). This elution solution was removed and the PVDF was washed twice with 60 microlitres of TFA (0.1% w/v). The digestion and elution solutions were pooled together with two final washes and this mixture was separated by two-dimensional reverse phase
25 -HPLC and sequence determination performed.

Immunoblotting

PVDF membranes were first stained to visualise proteins, following which the immunodetection was undertaken. This
30 allowed matching of proteins detected with ECL against those detected with the non-specific protein stain through computer comparison of both images. The mechanical strength of PVDF was also exploited as the

same 2-D gel can be used many times for different antibodies.

5 The whole procedure was carried out in a rotating oven at room temperature. The use of a nucleic acid glass hybridiser tube minimised the volumes and costs.

- 10 • The membranes were blocked in 10ml of a solution of PBS (pH 7.2) and non-fat dry milk (5% w/v) for 30 min.

- 15 • The membranes were then incubated in 10ml of a solution containing PBS-"Tween" 20 (0.5% v/v), non-fat dry milk (5% w/v) and the primary antibody/antibodies (1:100 or greater, depending on the antibody) for 2 hours.

- 20 • Three quick rinses were performed with 10 ml of PBS-"Tween" 20 (0.5% v/v) and then the membranes were washed for 3 x 10 min with 10ml of PBS-"Tween" 20 (0.5% v/v).

- 25 • The membranes were incubated in 10ml of a solution containing PBS-"Tween" 20 (0.5% v/v), non-fat dry milk (5% w/v) and the secondary peroxidase-conjugated antibody (1:1000; for example, if the primary antibody was sheep anti-mouse, then goat anti-sheep IgG was used as the secondary antibody) for 1 hour.

- 30 • Three quick rinses were performed with 10ml of PBS-"Tween" 20 (0.5% v/v) and then the membranes were washed for 5 x 10 min with 10ml of PBS-"Tween" 20

(0.5% v/v).

- After the last wash, the membranes were transferred to a clean glass plate and covered with 10ml of developing solution (for example ECL from Amersham International or Roche Diagnostics) for 2 min.
- The excess developing solution was drained, the membranes were wrapped in "Saran" film and fixed in an X-ray film cassette with the proteins facing up.
- An X-ray film was then exposed in a dark room for a few seconds or up to several minutes.

Peptide mass fingerprinting

The 2-DGE method was repeated, but using a Coomassie blue stain. The 2-DGE spots were destained with 100 microlitres of 30% acetonitrile in 50mM ammonium bicarbonate at 37°C for 45 min. The supernatant was discarded and the gel spots dried in a "SpeedVac" for 30min. The gel spots were rehydrated with 25 microlitres of a solution containing 0.2 micrograms of porcine trypsin and 50mM ammonium bicarbonate for 2 hours at 35°C and dried again for 30 min. Twenty microlitres of a solution of 50% of acetonitrile and 0.1% of TFA was added to the spots and sonicated for 10 min. Two microlitres of the supernatants was loaded in each well of a 96 or 400 MALDI target plate. The samples were air-dried. Then 2 microlitres of a solution containing 4mg/ml of alpha-cyano-4-hydroxycinnamic acid, 50% acetonitrile and 0.1% TFA was added to each well and air-dried.

The peptide mixtures were analysed by matrix-assisted

laser desorption/ionisation time-of-flight mass spectrometer (Perseptive Biosystems Voyager Elite MALDI-TOF-MS) with a nitrogen laser (337nm) and operated in reflectron delayed extraction mode.

5

Peptide identification has been carried out using "PeptIdent" (<http://www.expasy.ch/sprot/peptident.html>). It is a tool that allows the identification of proteins using pI, relative molecular mass and peptide mass fingerprinting data. Experimentally measured, user-

10 specified peptide masses were compared with the theoretical peptides calculated for all proteins in the SWISS-PROT/TREMBL databases.

15

MS/MS sequencing

When protein identification was not successful with the peptide mass fingerprinting procedure, the supernatant of digested spots was desalted in "ZipTip" C18 pipette tips (Millipore) and eluted with 50% acetonitrile and 0.1% TFA. Peptides were applied by nanoflow (in-house nanospray) sample introduction to a tandem mass spectrometer that consists of two quadrupoles and an orthogonal time of flight tube (Q-TOF) from MicroMass (UK). Fragment ion spectra were interpreted with the

20 MOWSE database search

25 (<http://www.seqnet.dl.ac.uk/mowse.html>).

Data management: the mouse SWISS-2-DPAGE database

SWISS-2-DPAGE is an annotated 2-D-PAGE database in which

30 all the data are easily retrieved by computer programs and stored in a format similar to that of the SWISS-PROT Protein Sequence Database, one of the most updated and annotated protein sequence databases presently available.

The SWISS-2-DPAGE database assembles data on proteins identified on various 2-D-PAGE maps. Each SWISS-2-DPAGE entry contains data on one protein, including mapping procedures, physiological and pathological data and bibliographical references, as well as several 2-D-PAGE images showing the protein location. Cross-references are provided to SWISS-PROT and, through the latter, to other databases (EMBL, Genbank, PROSITE, OMIM, etc.). The database has been set upon the ExpASY World Wide Web server (<http://www.expasy.ch/>). Worldwide, scientists using similar 2-D-PAGE protocols (immobilised pH gradient as first dimensional separation) are now able to compare their images with SWISS-2-DPAGE maps.

The references mentioned herein are all expressly incorporated by reference.

Claims:

1. A method of screening an agent to determine its usefulness in treating a condition characterised by pancreatic islet or β -cell dysfunction, the method comprising:

(a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of pancreatic islet or β -cell function;

(b) obtaining a sample of relevant tissue taken from, or representative of, a subject having reduced pancreatic islet or β -cell function, who or which has been treated with the agent being screened;

(c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the tissue from, or representative of, the treated subject; and,

(d) selecting or rejecting the agent according to the extent to which it changes the expression of the differentially expressed protein or proteins in the treated subject having reduced pancreatic islet or β -cell function.

2. The method of claim 1, wherein the agent is selected if it converts the expression of the differentially expressed protein towards that of a subject having more normal pancreatic islet or β -cell function.

3. The method of claim 1 or claim 2, wherein the agent is selected if it converts the expression of the protein or proteins to that of the normal subject.

4. The method of any one of claims 1 to 3, wherein the

pancreatic islet or β -cell dysfunction is a result of a disorder which causes a reduction in pancreatic islet or β -cell mass and/or a reduction in a pancreatic islet or β -cell biological activity.

5

5. The method of any one of the preceding claims, wherein the paradigm is based on tissue from non-insulin dependent diabetic subjects and normal subjects.

10

6. The method of claim 1, wherein the paradigm is based on animals which are models of non-insulin dependent diabetes as a result of a genetic mutation such as ob/ob, db/db, agouti, fat, fa/fa together with lean littermates.

15

7. The method of claim 1, wherein the paradigm is based on animals in which islet or β -cell dysfunction is exacerbated by dietary treatment.

20

8. The method of claim 1, wherein the paradigm is based on desert rodents such as spiny mice or sand rats which develop diabetes on normal laboratory diets but remain normoglycaemic on their natural diet.

25

9. The method of claim 1, wherein the paradigm is based on animals with gender selective differences in pancreatic islet or β -cell mass.

30

10. The method of claim 1, wherein the paradigm is based on closely related animals, such as C57BI/6 and C57BI/Ks mice which show differences in pancreatic islet or β -cell mass.

11. The method of claim 1, wherein differential levels

of islet cell or β -cell mass or function are induced by modifying the diet of pregnant animals or by comparing pregnant and non-pregnant animals in the paradigm.

5 12. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of levels of pancreatic islet or β -cell function comprise normal subjects and subjects having reduced levels of pancreatic islet or β -cell function.

10 13. The method of claim 12, wherein the reduced levels of pancreatic islet or β -cell function are the result of non-insulin dependent diabetes (type 2 diabetes), syndrome X or insulin resistance syndrome or gestational
15 diabetes.

14. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of levels of pancreatic islet or β -cell function
20 comprise normal subjects and subjects having a higher than normal level of pancreatic islet or β -cell function.

15. The method of claim 14, wherein the higher levels of pancreatic islet or β -cell function in the subjects are
25 obtained by treatment with an insulin sensitiser drug, dietary restriction or exercise.

16. The method of claim 15, wherein the insulin sensitising drug is thiazolidinedione insulin sensitiser.

30 17. The method of claim 16, wherein the thiazolidinedione insulin sensitiser is rosiglitazone (BRL 49653).

18. The method of claim 15, wherein the insulin sensitiser drug is a non-thiazolidinedione acting as an agonist or partial agonist of the PPAR gamma nuclear receptor.

5

19. The method of claim 18, wherein the insulin sensitiser drug is a β_3 -adrenoceptor agonist.

10

20. The method of claim 14, wherein the subjects having a higher than normal level of pancreatic islet or β -cell function are pregnant animals.

15

21. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise normal subjects and subjects having reduced pancreatic islet or β -cell function.

20

22. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise:

(a) normal subjects and subjects having reduced pancreatic islet or β -cell function; and,

25

(b) subjects having reduced pancreatic islet or β -cell function which have not been treated with the agent and subjects having reduced pancreatic islet or β -cell function which have been treated with the agent.

30

23. The method of claim 22, wherein the differential levels of protein expression are not observed in normal subjects who have and have not been treated with the agent.

24. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise:

(a) normal subjects who have and have not been treated with the agent; and,

(b) subjects having reduced pancreatic islet or β -cell function who have and have not been treated with the agent.

25. The method of claim 24, wherein the differential levels of protein expression are not observed in normal subjects and subjects having reduced pancreatic islet or β -cell function, both groups of subject being untreated with the agent.

26. The method of any one of the preceding claims, wherein the paradigm is established using two-dimensional gel electrophoresis carried out on the relevant tissue or a protein-containing extract thereof.

27. The method of any one of the preceding claims, further comprising the step of isolating a differentially expressed protein identified in the method.

28. The method of claim 27, further comprising the step of characterising the isolated protein.

29. The method of claim 28, further comprising using the protein in an assay for specific binding partners of the protein.

30. The method of claim 28, further comprising using the protein in an assay to screen for agonists or antagonists

of the protein.

5 31. The method of any one of claims 1 to 30, wherein the agents or proteins are screened using a high throughput screening method.

10 32. A method of making a pharmaceutical composition which comprises having identified an agent using the method of any one of claims 1 to 26, the further step of manufacturing the agent and formulating it with an acceptable carrier to provide the pharmaceutical composition.

15 33. Use of an agent identified by the method of any one of claims 1 to 26 for the preparation of a medicament for the treatment of a condition characterised by islet or β -cell dysfunction.

20 34. The use of claim 33 wherein the condition is non-insulin dependent diabetes (type 2 diabetes), syndrome X or insulin resistance syndrome or gestational diabetes.

25 35. A method of treating a condition characterised by islet or β -cell dysfunction in a patient, the method comprising administering a therapeutically or prophylactically effective amount of such an agent identified by a method of any one of claim 1 to 26 to the patient.

30 36. The method of claim 35, wherein the pancreatic islet or β -cell dysfunction is a result of non-insulin dependent diabetes or type 2 diabetes, syndrome X or insulin resistance syndrome or gestational diabetes.

37. A method of determining the nature or degree of pancreatic islet or β -cell dysfunction in a human or animal subject, the method comprising:

5 (a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of pancreatic islet or β -cell function;

(b) obtaining a sample of the tissue from the subject;

10 (c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the sample; and

15 (d) relating the determination to the nature or degree of the pancreatic islet or β -cell function by reference to a previous correlation between such a determination and clinical information.

38. The method of claim 37, wherein the sample is a tissue sample or body fluid sample or urine.

20 39. The method of claim 37 or claim 38, wherein in the paradigm at least four proteins are differentially expressed, providing a multi-protein fingerprint of the nature or degree of the pancreatic islet or β -cell
25 dysfunction.

40. The method of any one of claims 37 to 39 which further comprises determining an effective therapy for treating the pancreatic islet or β -cell dysfunction.

30 41. A method of treatment by the use of an agent that will restore the expression of one or more differentially expressed proteins in the pancreatic islet or β -cell

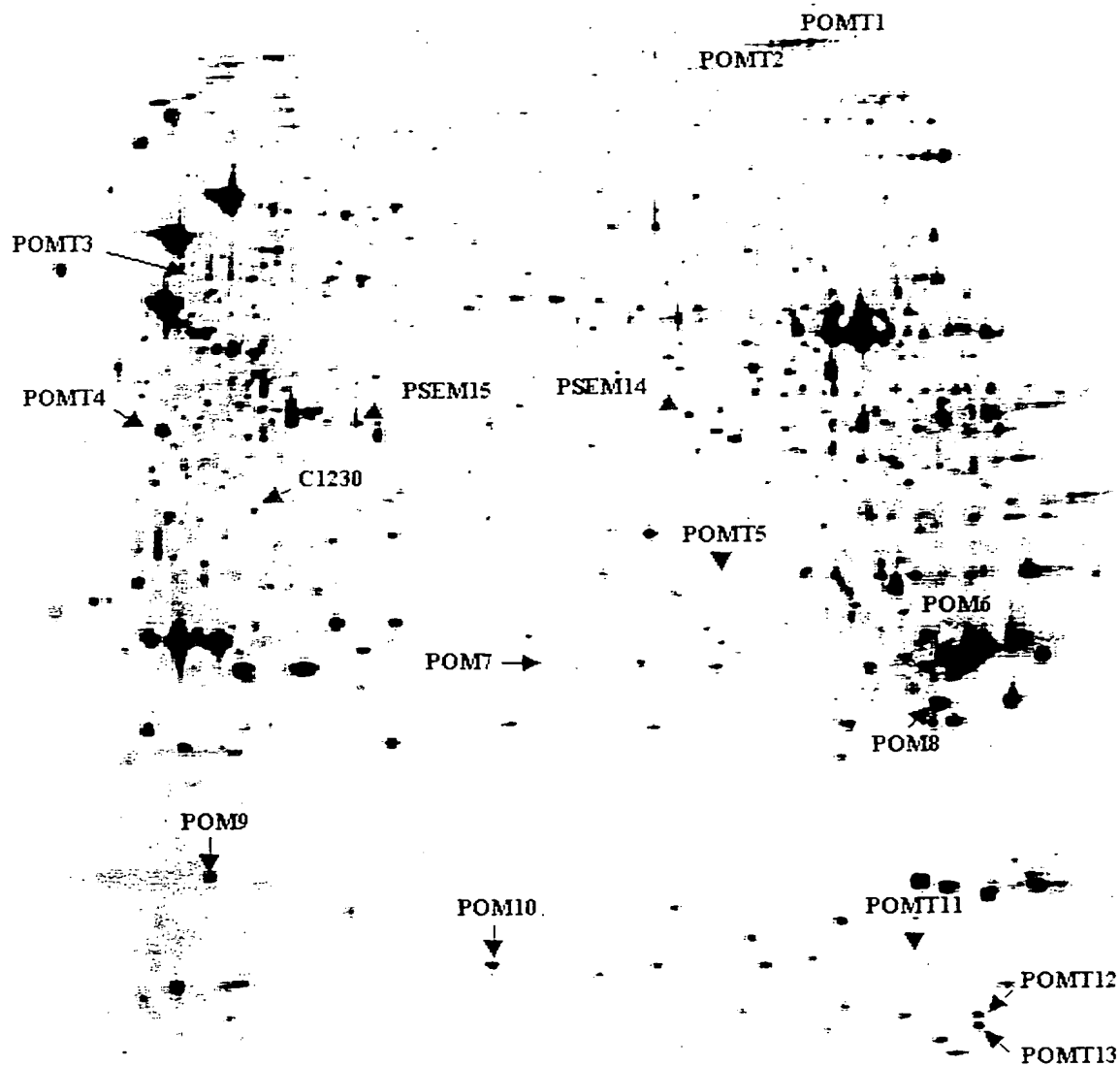
dysfunction state to that found in the normal state in order to prevent the development of non-insulin dependent diabetes in a pre-diabetic subject.

5 42. A method whereby the pattern of differentially
expressed proteins in a tissue sample or body fluid
sample or urine of an individual with pancreatic islet or
10 β -cell dysfunction is used to predict the most
appropriate and effective therapy to alleviate the
pancreatic islet or β -cell dysfunction state and to
monitor the success of that treatment.

15 43. A method of claim 42 whereby the pancreatic islet or
 β -cell dysfunction state is non-insulin dependent
diabetes or type 2 diabetes.

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Figure 1



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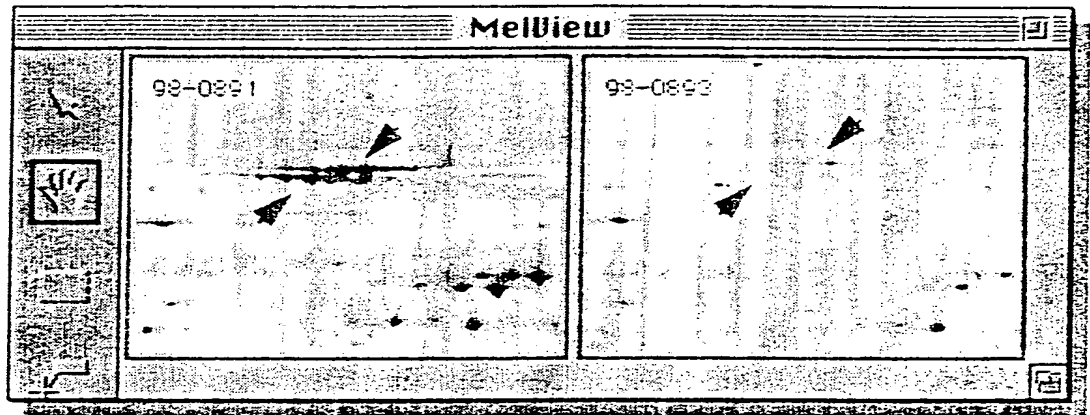
Figure 2

Pancreatic islet molecular markers related to the obesity state (POM)

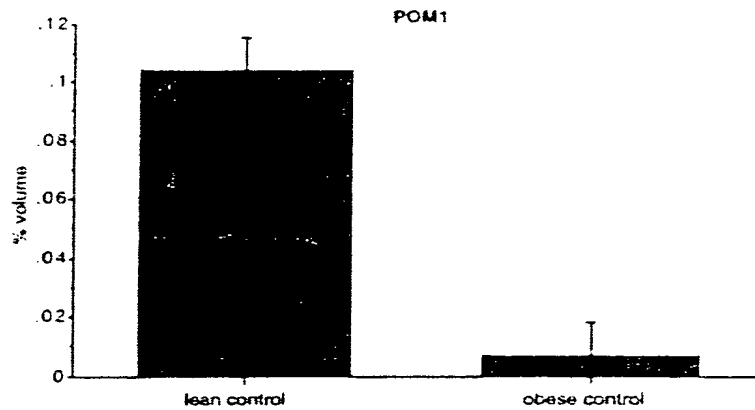
Lean

Obese

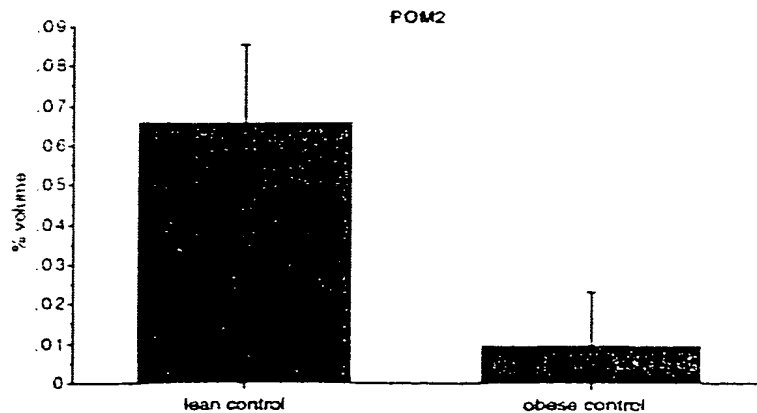
POM1
+
POM2



POM1



POM2



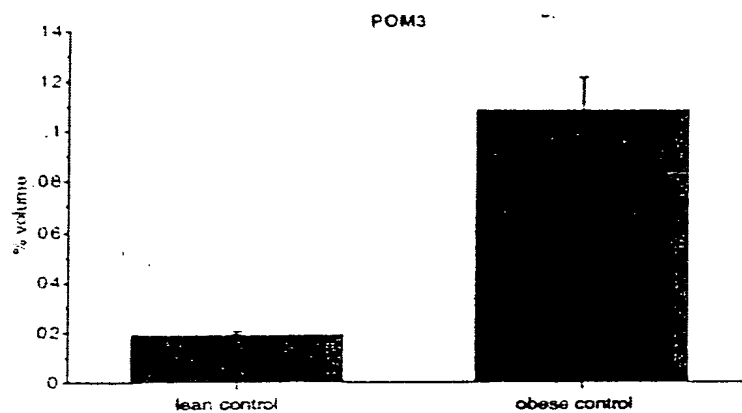
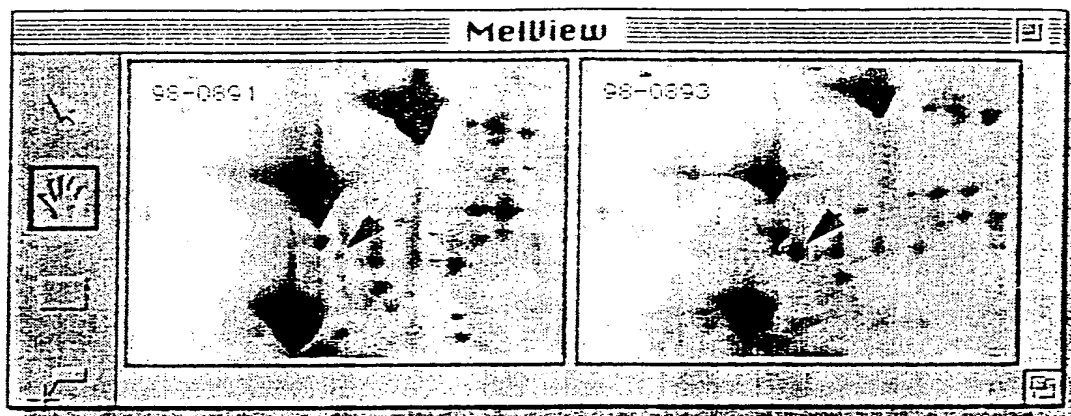
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Figure 3

Lean

Obese

POM3



POM4

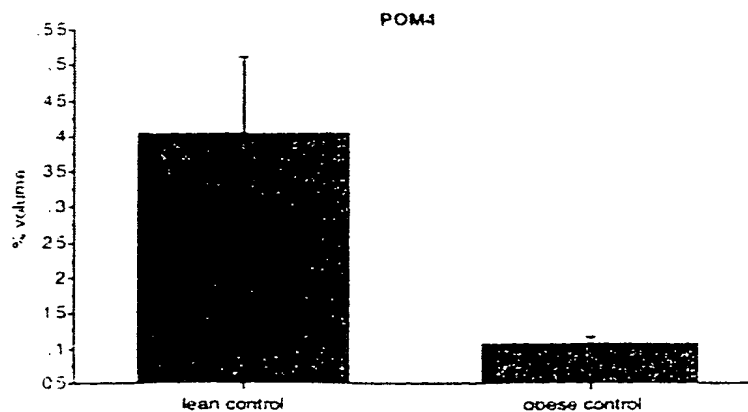
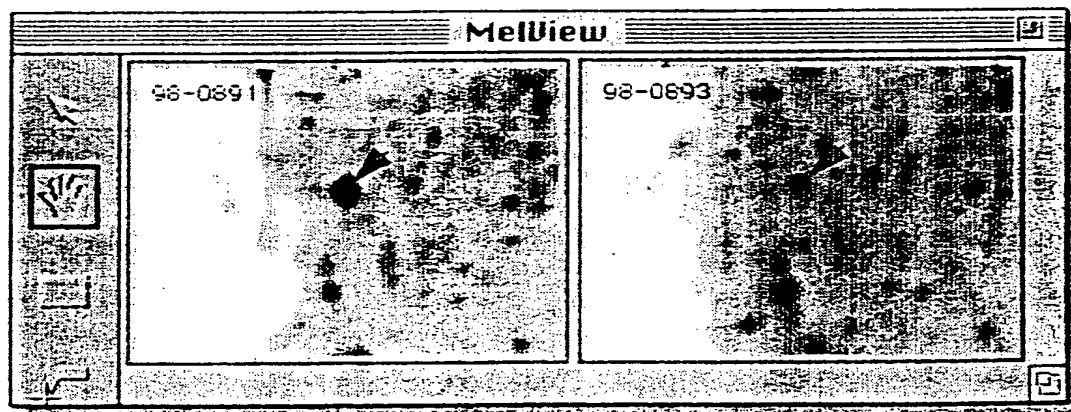


Fig 5A

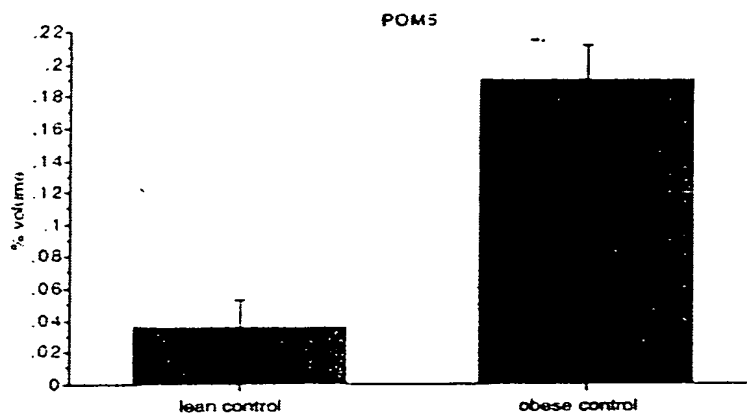
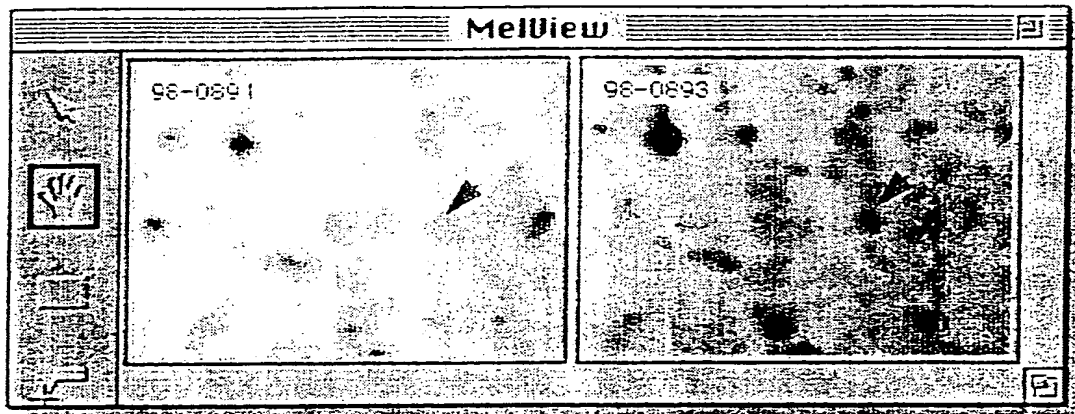
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Figure 4

Lean

Obese

POM5



POM6

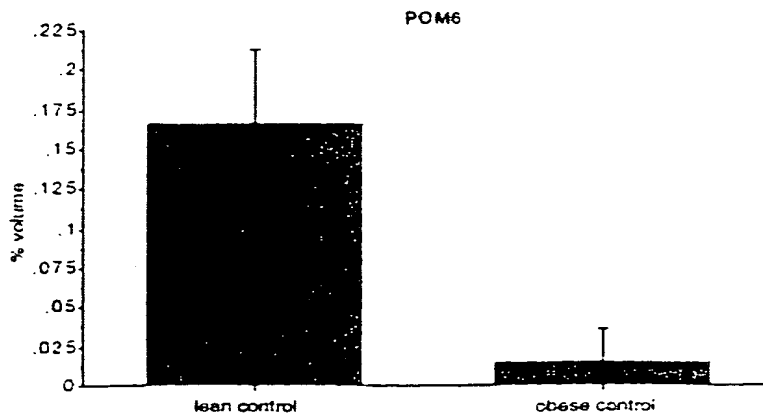
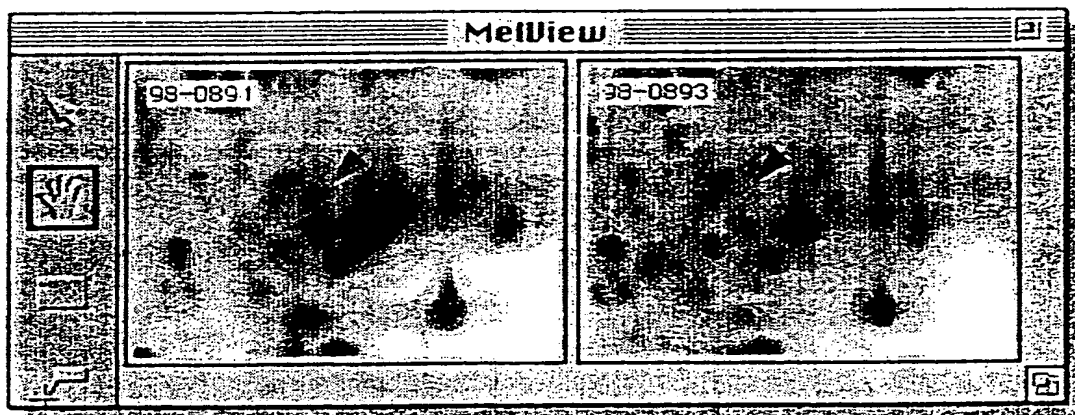


Fig 5A

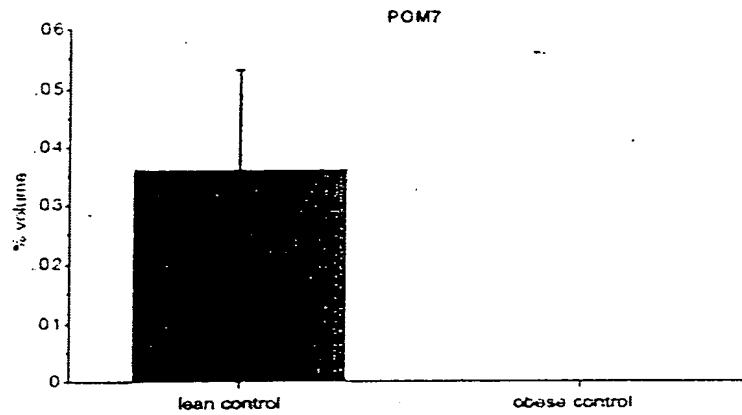
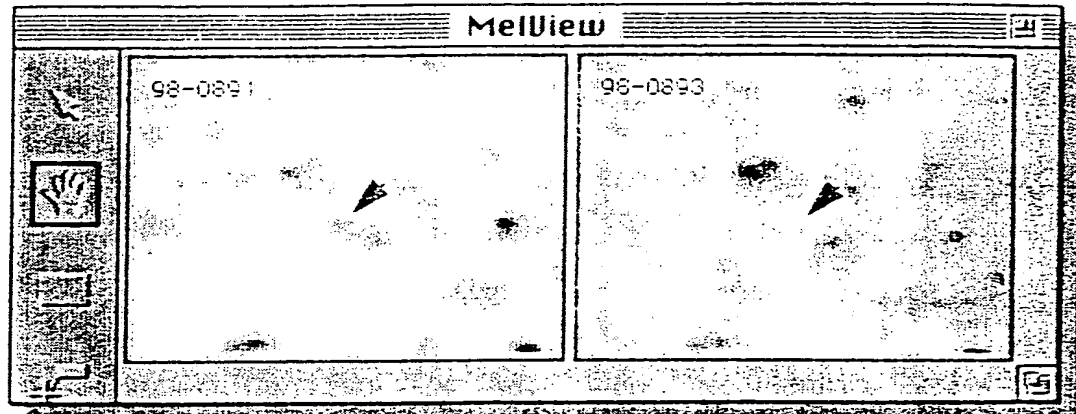
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Figure 5

Lean

Obese

POM7



POM8

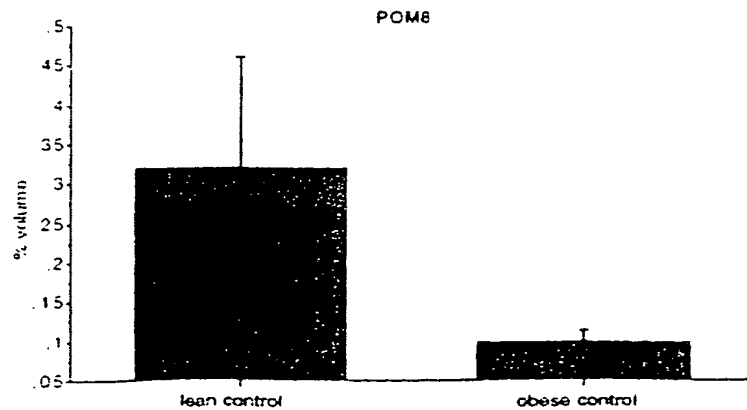
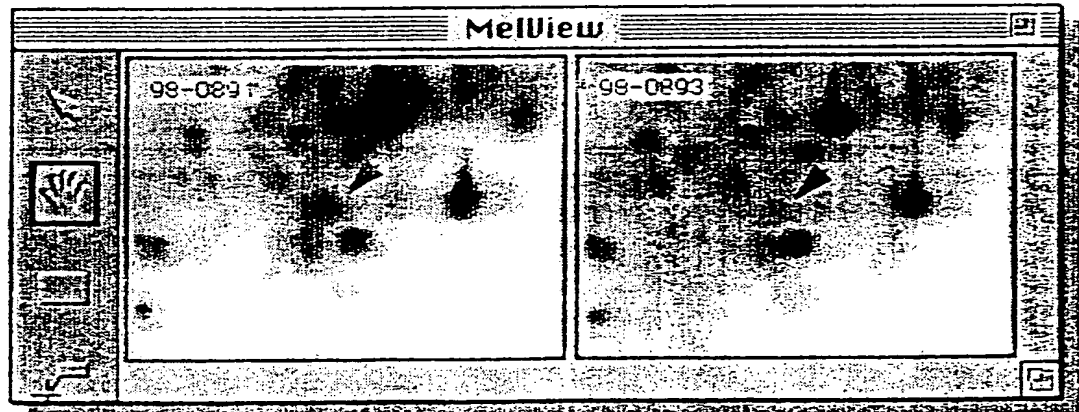


Fig 5A

5114

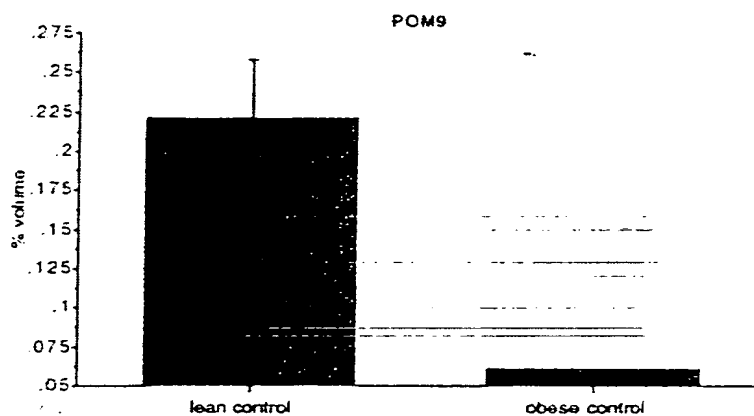
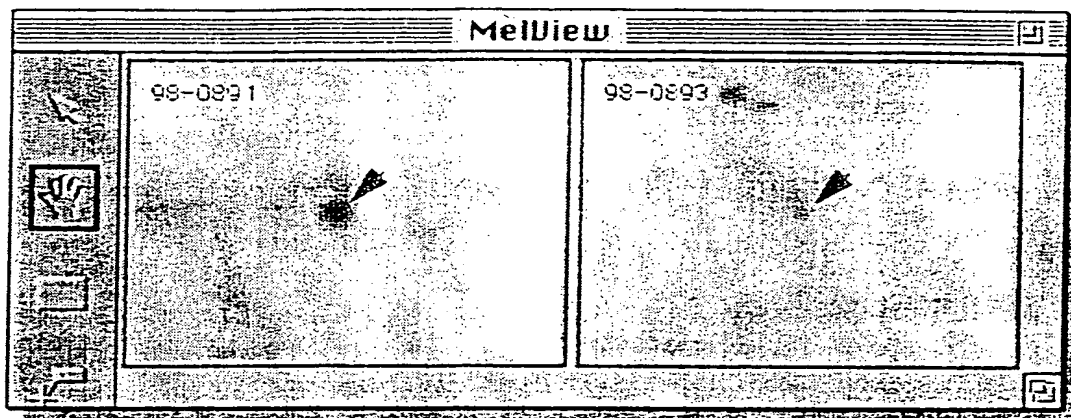
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Figure 6

Lean

Obese

POM9



POM10

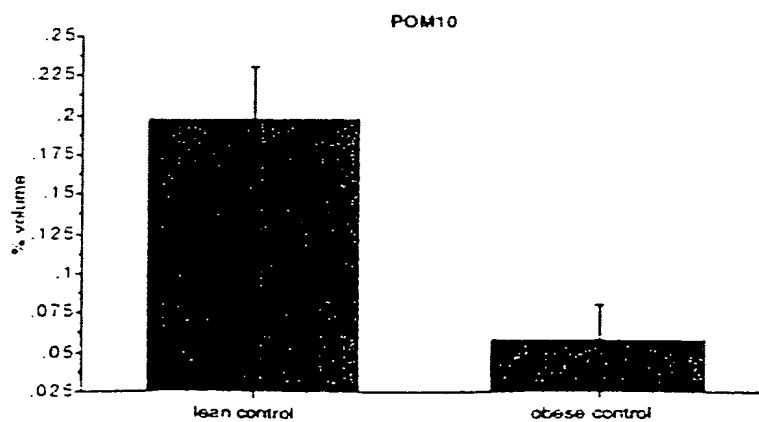
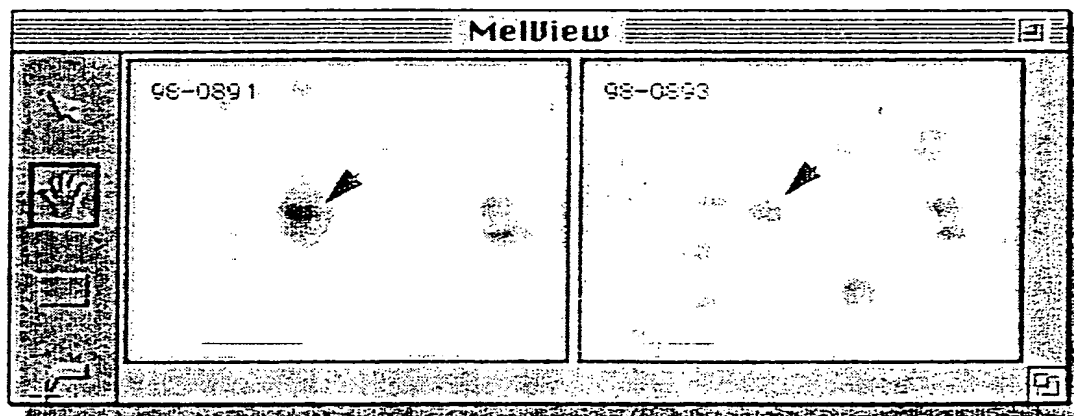


Fig 5A

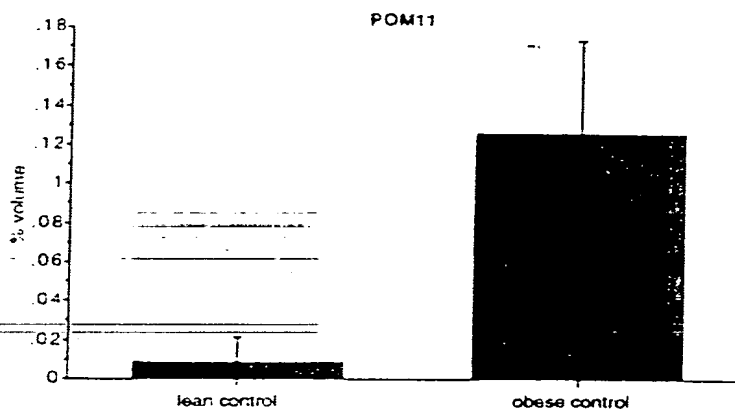
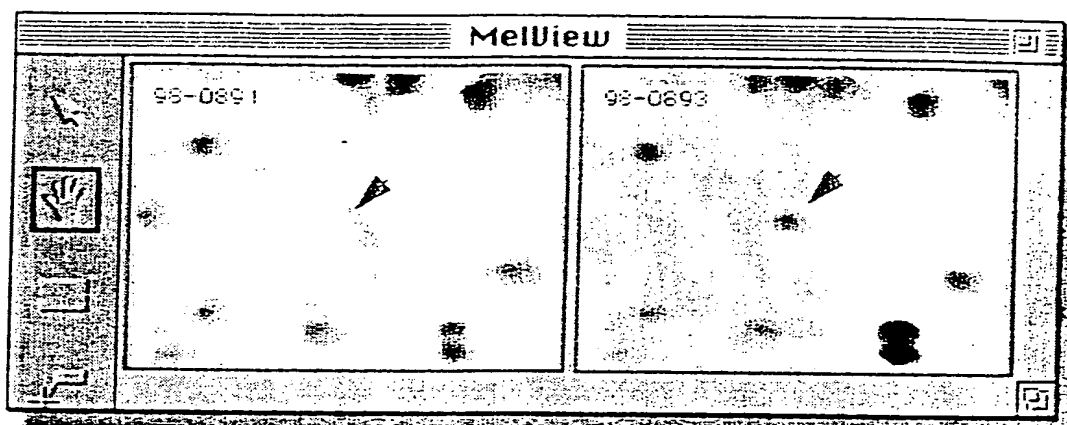
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Figure 7

Lean

Obese

POM11



POM12
+
POM13

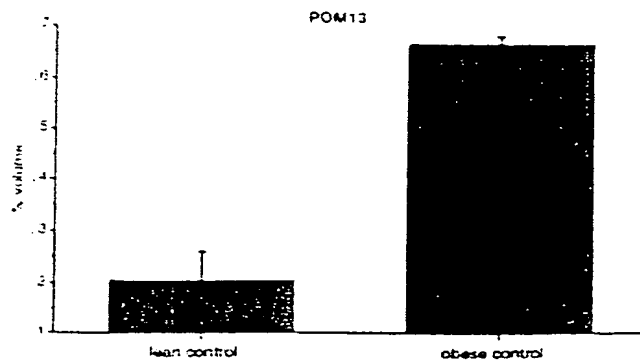
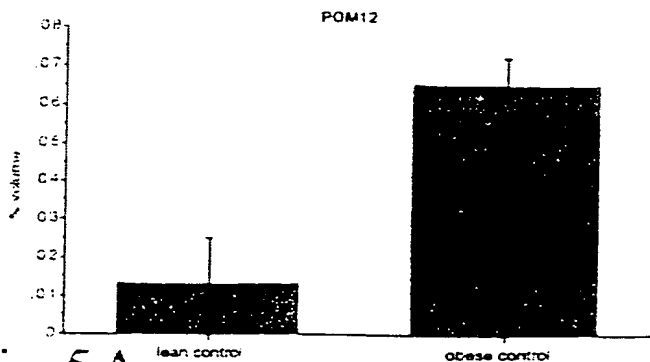
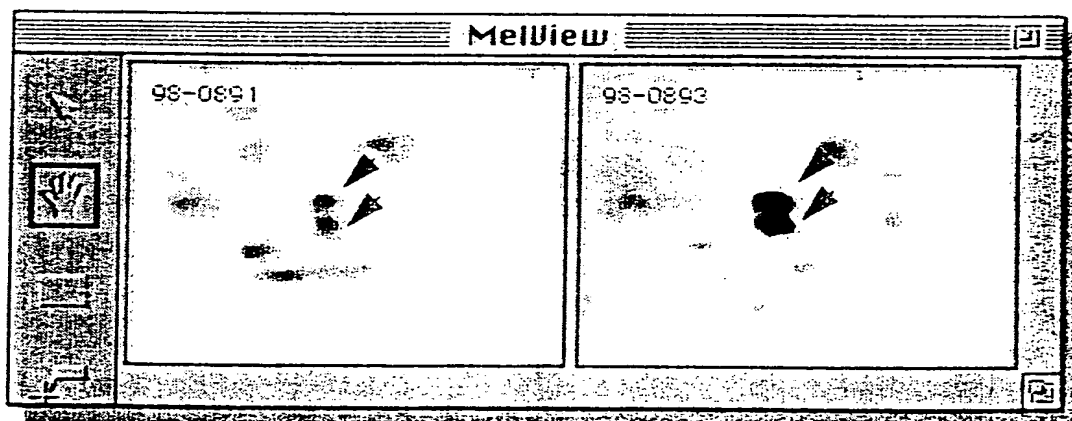


Fig 5A

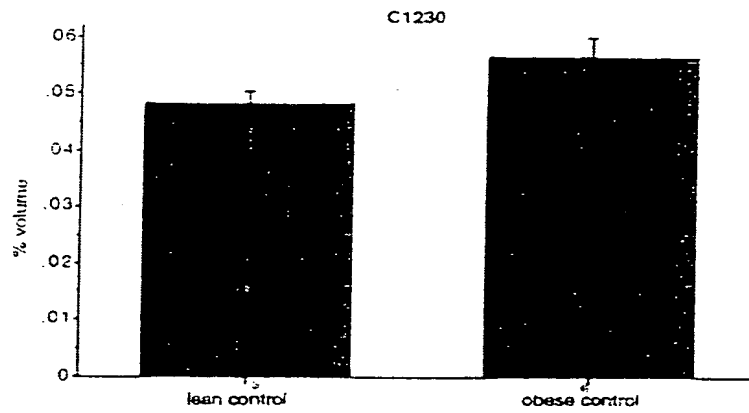
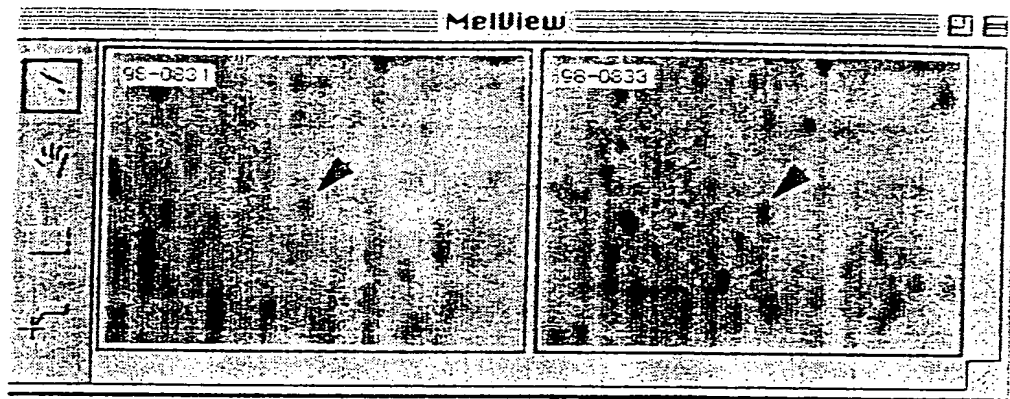
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Figure 8

Lean

Obese

Control
1230



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Figure 9

Pancreatic islet markers related to the obesity state
regulated by rosiglitazone (POMT)

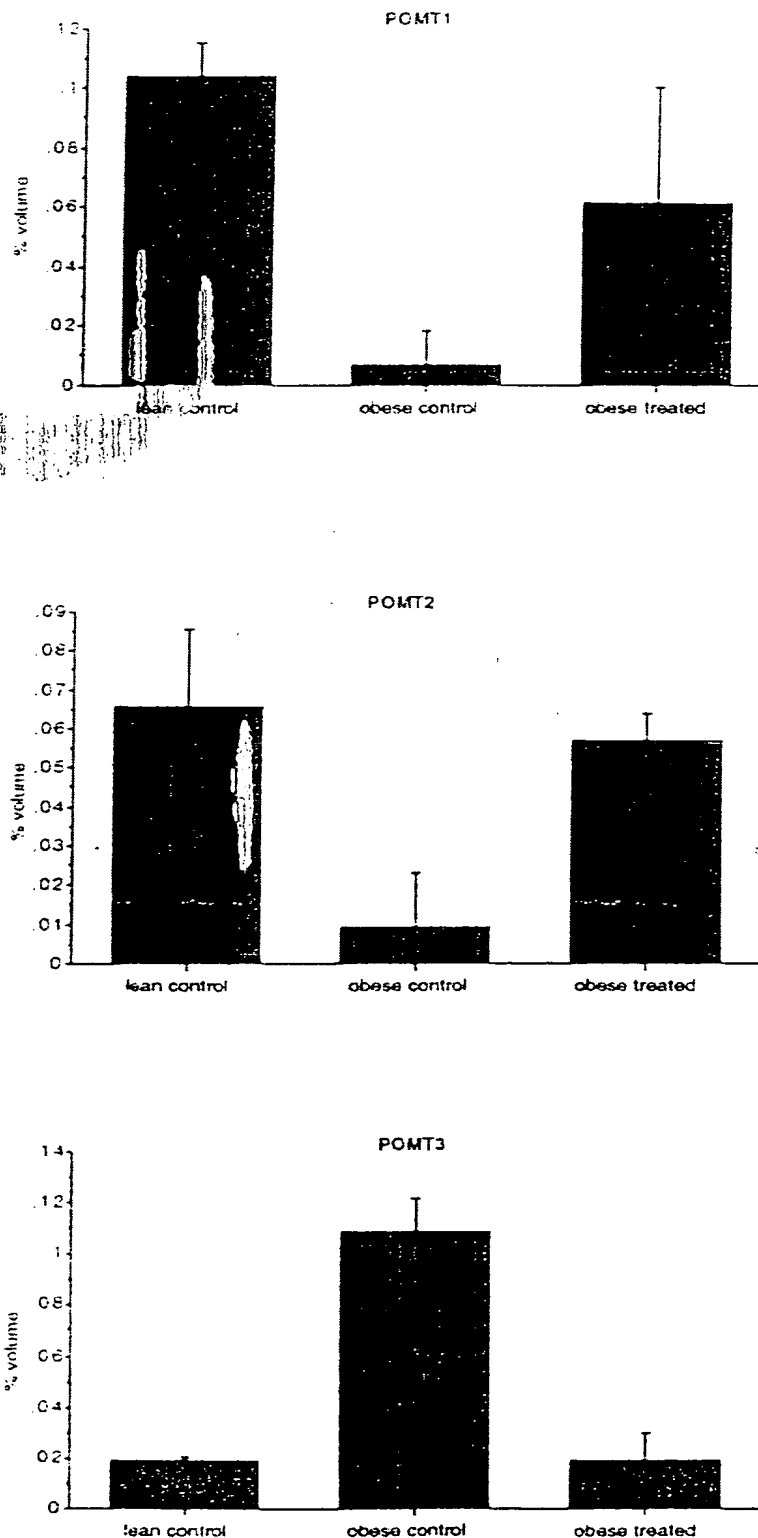
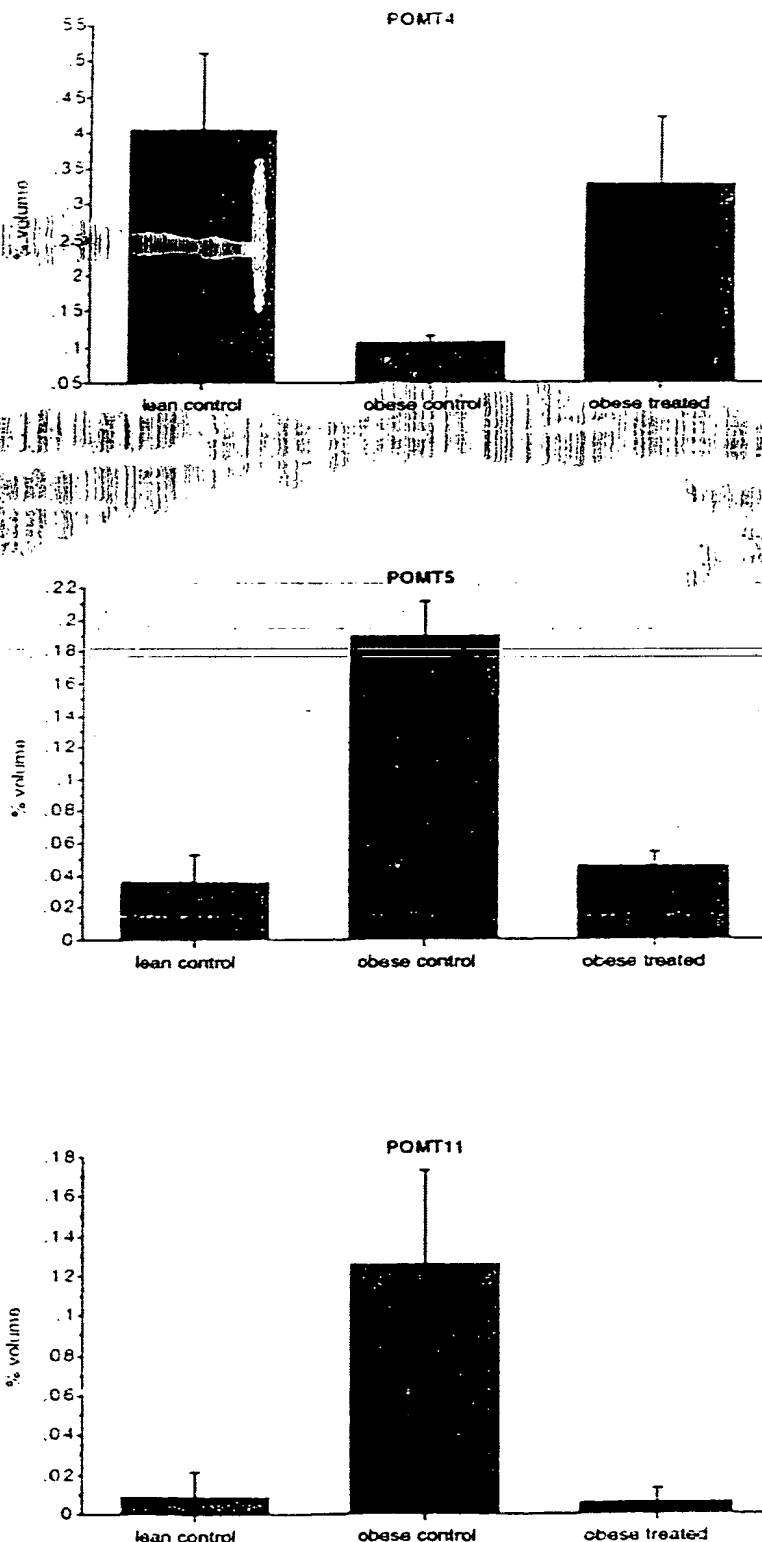


Fig 5B

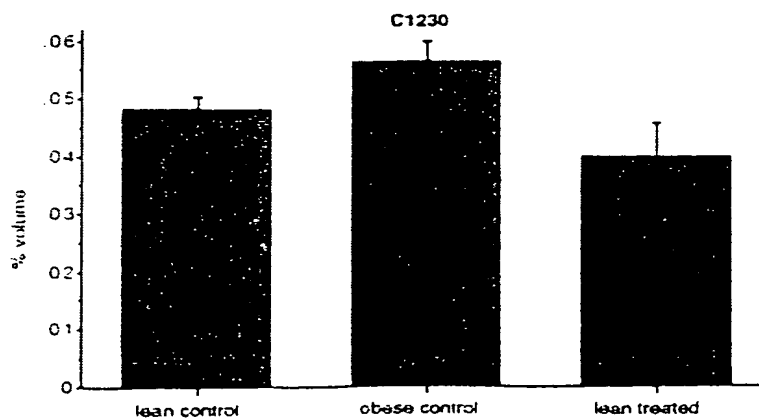
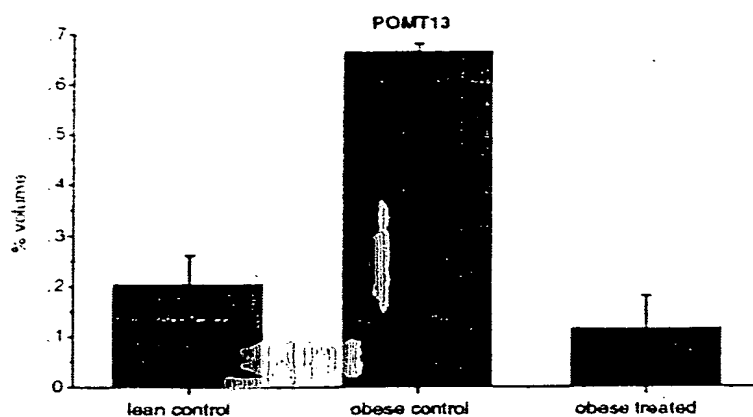
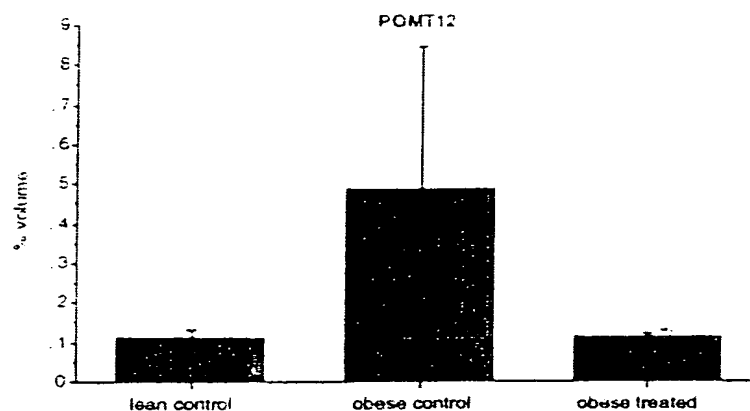
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Pancreatic islet markers related to the obesity state regulated by rosiglitazone (POMT)



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Pancreatic islet markers related to the obesity state regulated by rosiglitazone (POMT)



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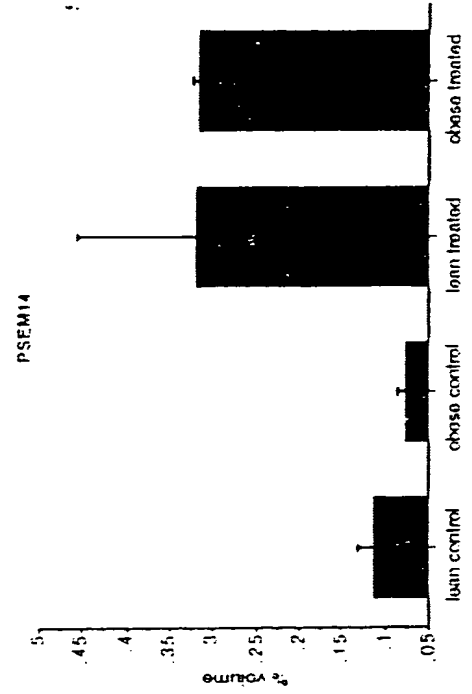
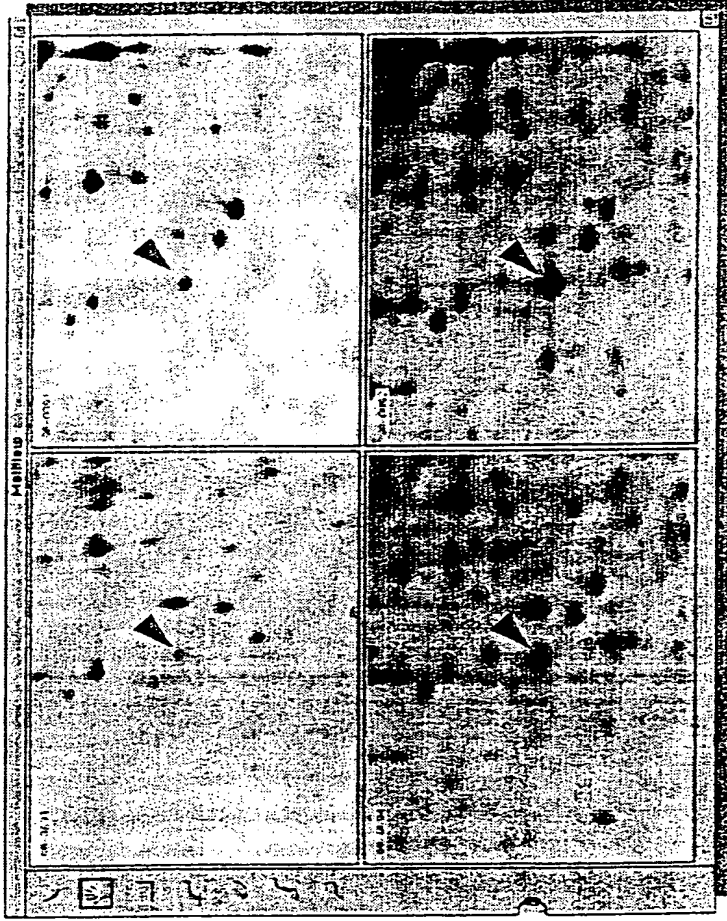
Pancreatic islet rosiglitazone side effect markers (PSEM)

Lean
(control)

Lean
(treated)

Obese
(control)

Obese
(treated)

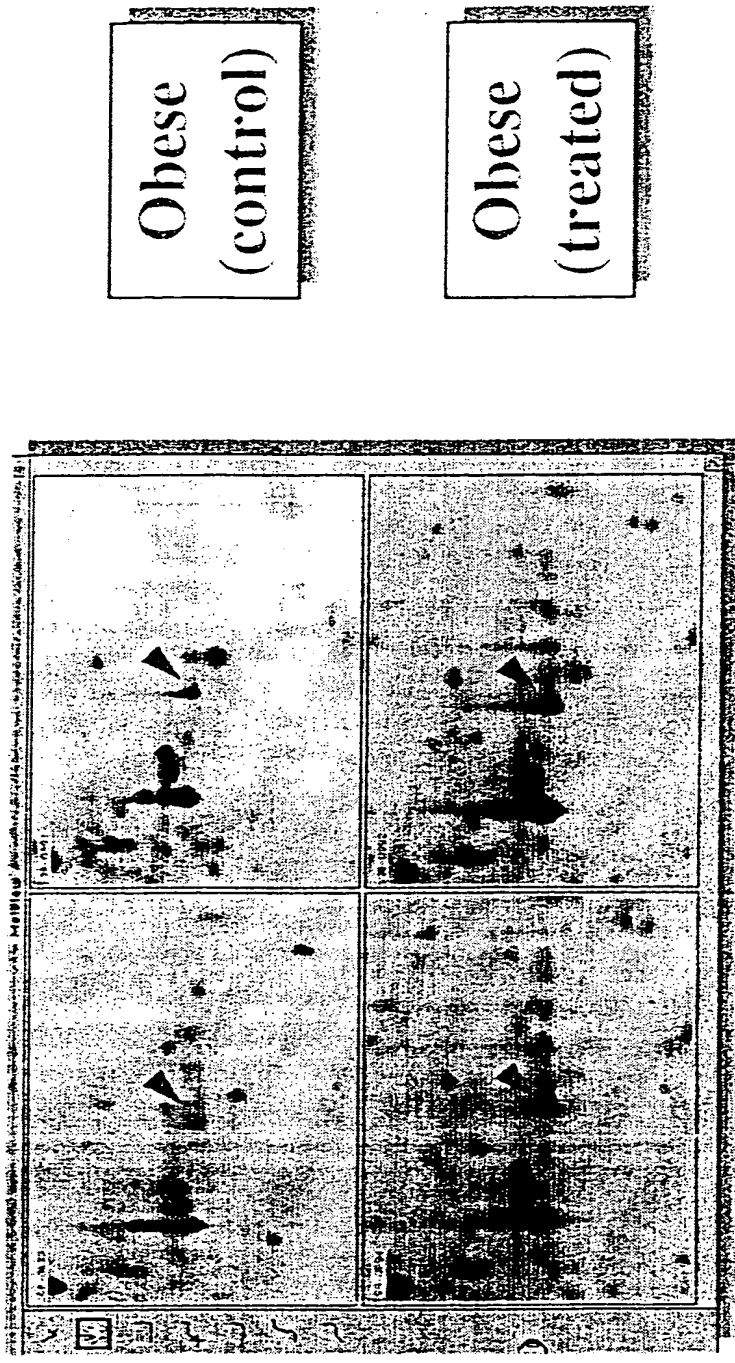


PSEM14

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Pancreatic islet rosiglitazone side effect markers (PSEM)

Figure 13

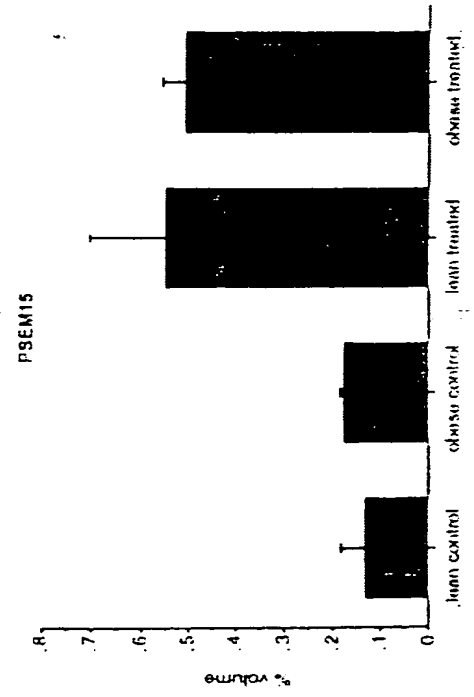


Lean
(control)

Lean
(treated)

Obese
(control)

Obese
(treated)



PSEM15

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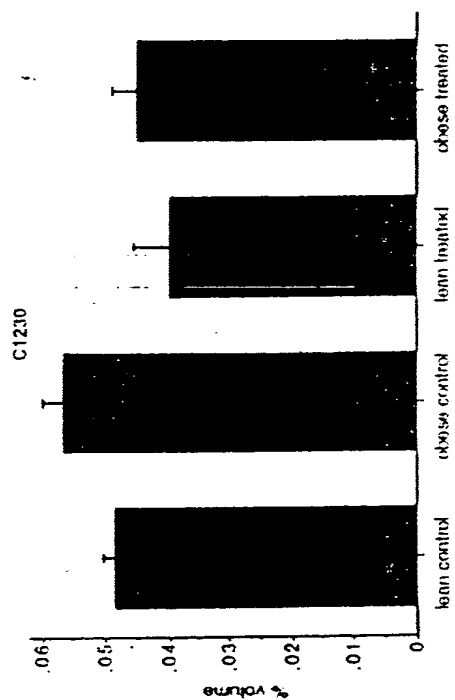
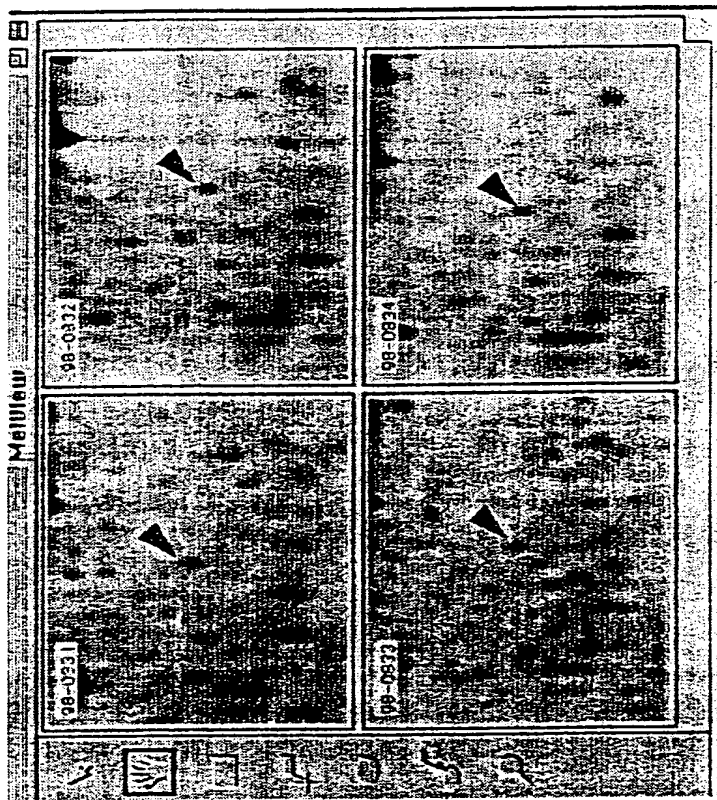
Pancreatic islet rosiglitazone side effect markers (PSEM)

Lean
(control)

Lean
(treated)

Obese
(control)

Obese
(treated)



Control
1230

Fig 5C

Figure 14

McDonald, Ellis

1/6/00

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